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THE BOTANICAL REVIEW

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THE MOVEMENT OF MATERIALS INTO PLANTS PART I. OSMOSIS AND THE MOVEMENT OF WATER INTO PLANTS

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INTRODUCTION

Numerous articles have been published on various phases of the movement of water into plants. The theoretical aspects of most such publications have been limited to explanations of specific experimental data. Only in text-books are comprehensive discourses presented, and frequently unity of thought is lost in the circuitous, interrelated discussions on several major physiological subjects. There is a need for a unified discussion of osmosis and its relation to water movement (water migration) into plants. The present theoretical treatment of the subject is presented in the hope of clarifying and extending present concepts in this important field of study. A new approach is attempted, citing examples, in accord qualitatively and quantitatively with experimental results, and based upon current definitions and principles of physics and physical chemistry (4, 7, 9, 11, 17-20, 35, 40, 43, 44, 61). A complementary treatise on the tendency for solute to move (solute migration) into plants will be given in Part II of this study.

THEORETICAL ASPECTS OF OSMOSIS

The association of the various factors tending to cause water to move through the plant may be concisely developed from basic laws of physical chemistry, as follows:

The Ideal Gas Law: A perfect gas system is completely defined by the relationship (35, p. 63):

$$PV = nRT, \text{ where} \quad (1)$$

P is the pressure, due to the kinetic energy of the molecules, here expressed in atmospheres; V is the volume, in liters; n is the number of mols of gas; R is a "constant" factor, 0.08205 liter-atmosphere per degree; T is the absolute temperature.

The Free Energy Concept: The fundamental principle underlying the movement of materials is that each molecule possesses a total internal energy equal to the sum of its internal kinetic and potential energies (18, p. 57), and the molal (or partial molal) free energy is equal to the product of the mean free energy of the particles and the number of particles in one mole. A system is subject to spontaneous change if there is any conceivable process whereby the internal energy of the constituent molecules can be effectively reduced. The action, here especially that concerned with translation of the particle in space—its escaping tendency—which could be produced by such a conceivable process, is determined by the internal free energy of the individual molecules. The free energy of the particles may be modified by any change in condition of the external environment.

Diffusion Defined: Diffusion is the process whereby one substance moves into or through another in response to a difference of partial molal free energy¹, tending toward equilibrium of escaping tendencies (35, pp. 179, 180).

Osmosis Defined: Osmosis is a process of diffusion of the component capable of free passage through a semipermeable membrane separating two components², only one of which is capable of free passage through the boundary (54a). An osmotic system is a system in which the process of osmosis takes place. The osmotic relations of the system are determined by the constituent factors or influences involved in the consummation of the process of osmosis.

A Semipermeable Membrane Defined: A semipermeable membrane, in an ideal osmotic system, is a limiting layer, of infinitesimal thickness, separating two components, only one of which is capable of free passage through the boundary.

¹ As defined by Lewis and Randall (35), to include also the possible influences of variables other than pressure, temperature and composition. The phrase "free energy" will be used hereinafter with the understanding that it refers to the partial molal free energy of the molecules of a component of the system. Osmosis may be attended by modification of the $p \times V$ product in either phase. The resultant action capacity between phases after any interval of time will in part depend on this product. Therefore, a free energy difference ($\bar{f} - f^\circ$) is here expressed as that, corrected for any significant $p \times V$ change in either phase, equivalent to a difference in "A" of Helmholtz (35, pp. 156-159).

² Since aqueous solutions are invariably involved in the two membrane-separated phases of biological systems, the terms "water" (solvent) and "solute" will be used, implying that the components are capable and incapable, respectively, of relatively free passage through the semipermeable membrane.

Osmotic Specific Free Energies Defined: Osmotic specific free energies (F) are any action capacities of water associated with an osmotic system, expressed in positive dimensions of $m L^{-1}, t^{-2}$, e.g., energy per unit volume, or pressure. An osmotic influx or efflux specific free energy is numerically equal to the difference in pressure, the osmotic pressure ($P = p - p^\circ$), on a medium necessary to render the escaping tendency of water in a given state equal to that of water in a reference state. This pressure difference is related to the free energy of the water molecules in a dilute solution of solute and solvent contained in either phase of an osmometer, through the approximate relation:

$$\mp P = \mp (p - p^\circ) = \frac{\pm (\bar{f} - f^\circ)}{v^\circ (1.013 \times 10^9)} = F \quad (2)^3$$

where \bar{f} is the free energy of water in a given state other than the standard or reference state, here expressed in erg units; f° is the molal free energy of water in its standard state, in ergs; v° is the molal volume of water in its standard state, in liters; 1.013×10^9 is the necessary conversion factor; p° is the pressure on the water in its standard or reference state, in atmospheres; and p is the pressure on the medium in the given state, necessary to make \bar{f} equal to f° , in atmospheres.

Since the molal volume of the component water in the standard state is a constant (here equal to 0.018 liter), it is evident from equation 2 that an osmotic influx or efflux specific free energy F , as well as an osmotic pressure P , is a measure of the quantity $(\bar{f} - f^\circ)$ (see 35, p. 214; 18, pp. 100-104). In this treatise specific free energies are used as measures of the differences in free energy of the water molecules due to any constituent osmotic influence, in order to deal with dimensions commensurate with those generally employed by the biologist (compare 44). However, the action capacities may be

³ Many physical chemists and biologists have related the escaping tendency of water through an interposed semipermeable membrane of an osmometer to the pressure difference $p - p^\circ$. However, since only one actual or exerted pressure is generally involved (i.e., hydrostatic) it is better, in order to avoid misconception, to express the osmotic values or action capacities, in terms of the differences in the free energy of the water molecules. This specific free energy F is expressed by the quotient $\pm \frac{(\bar{f} - f^\circ)}{v^\circ}$, related to, but not caused by, the pressure difference $p - p^\circ$ as set forth in equation 2 (compare (54a)).

NOTE: A bar above the symbols \bar{f} and \bar{v} indicate partial molal quantities. The circular zero as super-script to the symbols f° and v° indicate the standard state.

expressed in fundamental dimensions of energy mL^2t^{-2} , (e.g., erg or calorie units). For the units used in this dissertation, the conversion is accomplished through the relation $\pm (\bar{f} - f^\circ) = F (1.823 \times 10^7)$, where F is expressed in atmospheres, and $(\bar{f} - f^\circ)$ in ergs.

Osmotic Pressure Defined: Osmotic pressure is the pressure difference which must be applied to the two phases of an osmometer, under ideal conditions, to establish and/or maintain equilibrium of escaping tendency for water across the interposed semipermeable membrane (see 35, pp. 213–215). In equation 2 the osmotic pressure is represented by P , equal to the difference $p - p^\circ$ (see Figure 1). (See the section entitled "On the Origin of Osmotic 'Solute'

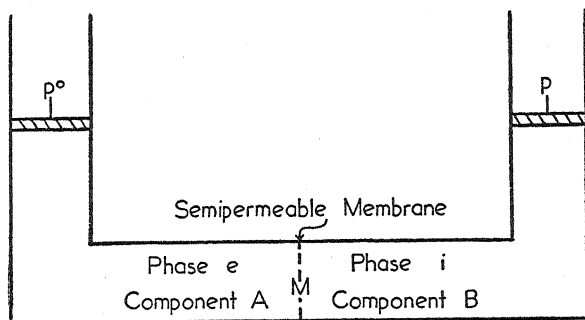


FIG. 1. Initial condition of an osmotic system in the ideal state.
 A = Component capable of free passage through the membrane.
 B = Component incapable of free passage through the membrane.
 M = Semipermeable membrane.

$$\pm \frac{(\bar{f} - f^\circ)}{v^\circ} = \mp p - p^\circ; \text{ for analysis, see text, page 4.}$$

Specific Free Energy", page 9, and compare with the solute specific free energy related to the pressure difference necessary to equilibrate the escaping tendencies for solute between two solution phases of an osmometer (Part II).)

Osmotic Metabolic Specific Free Energy Defined: In the plant, metabolic specific free energy, F_m , is any possible action capacity maintained directly through metabolism of the living organism which can be applied to tend to cause water to move, unilaterally, through the semipermeable membrane with or against the direction in which its concentration decreases⁴, by modification of the free energy of the water molecules.

⁴ It has been suggested that osmosis should be termed "anomalous" where the flow of water through the semipermeable membrane is governed by

Osmotic Non-metabolic Specific Free Energy Defined: In the plant osmometer non-metabolic specific free energy, F_{nm} , is any possible action capacity (exclusive of hydrostatic specific free energy, which see) not directly maintained through metabolism of the living organism, which can be applied to tend to cause water to move through the semipermeable membrane with or against the direction in which its concentration decreases, by modification of the free energy of the water. Adsorption and imbibition are two typical processes or influences affording an action capacity of this type through lowering of the free energy of the water molecules at colloidal surfaces and within imbibants. Such processes imply the presence of matter other than molecules of solvent and solute in solution.

Osmotic "solute" specific free energy is one manifestation of a possible non-metabolic osmotic specific free energy, but because of its primary importance in the osmotic system it is discussed as a separate factor for water flow.

Osmotic "Solute" Specific Free Energy Defined: Osmotic "solute" specific free energy, F_s , is the action capacity tending to cause water to move through the semipermeable membrane either into a component incapable of free passage through the semipermeable membrane or into a solution of the two components (solvent water and solute). The water flows across the boundary into a solution within which the free energy of the molecules of the water has been lowered in relation to the concentration of the solute in the solution. In an ideal osmotic system the relationship between

energies supplementing the osmotic "solute" specific free energy difference, as determined by the inequality of the solute concentration between the two phases of the osmometer. Anomalous osmosis is then designated "positive" where the flow is with the direction in which the concentration of water decreases; "negative" where the transport is against the direction in which the concentration of water decreases (9). In view of our limited knowledge of the factors involved, the term "anomalous" may justly be applied to this unknown osmotic mechanism. However, in order to maintain a consistent and more expressive terminology, the following definitions are proposed. "Metabolic specific free energy" tends to cause or maintain "osmotic, metabolic diffusion of water", the tendency toward flow being governed by energy supplementing the osmotic "solute" specific free energy difference, as determined by the inequality of the solute concentration between the two phases of the osmometer. The flow may be characterized as an "osmotic metabolic simple diffusion" where, and while, the flow is with the direction in which the concentration of water decreases; similarly, "osmotic metabolic accumulation" where the transport is against the direction in which the concentration of water decreases.

a measure of this specific free energy and the volume within either of the two phases involved, shows a formal resemblance to the ideal gas law (equation 1), *viz*:

$$F_s = \frac{n}{V} \cdot RT \quad (3)^5$$

where F_s is the specific free energy of the water, dimensionally and mathematically equal to the osmotic pressure, related to a difference in solute concentration in the two phases of the osmometer; V is the volume of solution; and n is the number of mols of solute in the solution (26).

Since $\frac{n}{V} = C$, where C is the volume molar (molarity) or, better, the weight molar (molality) concentration of solute in the solution,

$$F_s = CRT \quad (3b)$$

Assuming temperature changes are nil, the osmotic "solute" specific free energy may be measured as equal to the pressure which must be applied to produce a condition such that there is no tendency for the water to flow in either direction across the semipermeable boundary (see equation 2 and Figure 1). Employing the van't Hoff equation, 3 or 3a or 3b, as applicable in solution⁶, the osmotic "solute" specific free energy is a function of the concentration of solute in a solution. Thus RT is a coefficient for converting solute concentration into the related (antiphasically effective) osmotic "solute" specific free energy (see equation 3b).

Hydrostatic Pressure Defined: Hydrostatic pressure, P_h , is a pressure exerted uniformly in all directions from any point in a

⁵ Some investigators (see 35, p. 236), on theoretical and experimental grounds, prefer the equation

$$F_s = \frac{N}{v^0} \cdot RT \quad (3a)$$

where N is the number of mols of solute in 1 mol of solvent, and v^0 is the molal volume of the pure solvent.

⁶ If the equation of van't Hoff is used to define the specific free energy and concentration relations of an ideal solution, then departure from the ideal behavior must be interpreted with respect to such factors as are likely to influence the osmotic "solute" specific free energy. He introduced a coefficient, k , into the equation, to correct for all types of deviation from ideal behavior without recourse to their origin. The osmotic "solute" specific free energy may be modified by the degree of dissociation of the molecules of the solute into constituent ions (57, p. 55) and by hydration (45, pp. 102, 103), in aqueous systems. Further, corrections may be applied for non-solvent volume and for forces of attraction between molecules in solution (10). For examples of possible deviation from the ideal, see data of Molz (47), Morse (48) and others (57, pp. 51-56).

fluid. Other factors being constant, any change in the hydrostatic pressure within a phase will directly modify the free energy of the component molecules, proportionately. If the pressure on, and free energy of, the molecules at any point are increased, they are increased everywhere throughout the fluid by the same amount. More specifically, within the osmometer the hydrostatic pressure, Ph_1 , is the outwardly directed pressure of the inner component or solution on the membrane, which can be exhibited either by turgor in an inclosed phase or by a similar pressure in an open inner phase.

Such pressure, when positive, tends to cause water to move outward across the semipermeable boundary due to the increased free energy of its molecules in the inner phase of the osmometer (35, pp. 199, 243, 244). This pressure, positive or negative (18, p. 136), is equal to the algebraic result between that pressure (Ph_1') intrinsically associated with the medium in the inner phase of the reference system, caused by the restriction to expansion therein, and any extrinsic pressure (Ph_1'') which may arise due to the application of energy of extra-phasic origin, *i.e.*, from outside the reference system. In an open inner phase, within the plant, the intrinsic hydrostatic pressure (with reference to that at the upper surface of the inner phase taken as zero, capillarity excluded) is caused by the weight of the column of fluid alone, the restriction to expansion being associated with the increasing total force (gravitation involved) in the liquid column. The extrinsic force may be any partial solution pressure (positive or negative, and usually the latter in the plant) which may arise within the osmometer due to the application of an action capacity to either the external or internal phase. In an inclosed inner phase, the intrinsic pressure, numerically equal to the membrane (wall) pressure, is caused by the restriction to expansion applied through extension of the elastic encircling membrane of the reference cell. This pressure may be positive, zero or negative. Here, as in the open inner phase, the extrinsic force may be any partial solution pressure, positive or negative, which may arise within the osmometer due to the application of an action capacity to either the external or internal phase. With a reference cell, a special extraphasic energy may be involved. Where it is surrounded by other cells, as in a tissue, an intercellular (wall) pressure, positive or negative, is effective.

Hydrostatic Specific Free Energy Defined: Hydrostatic specific

free energy, Fh_1 , is that action capacity, caused by a hydrostatic pressure within an osmometer, which tends to cause water to move across the semipermeable boundary. Hydrostatic specific free energy is the resultant between the intrinsic hydrostatic specific free energy, Fh_1' , and any extrinsic hydrostatic specific free energy, Fh_1'' . These constituent specific free energies are related to the comparable constituent hydrostatic pressures.

Net Influx Specific Free Energy Defined: The net influx specific free energy, NIF, is the difference in action capacity between the algebraic sum of the specific free energies tending to cause water to move into the system and those tending to cause water to move out of the system. The net influx specific free energy is equal to the sum of the influx specific free energies diminished by the sum of the efflux specific free energies, *i.e.*, net influx specific free energy = $(\sum \text{influx specific free energies}) - (\sum \text{efflux specific free energies})$ or $NIF = \sum IF - \sum EF$. (4)

On the Origin of Osmotic "Solute" Specific Free Energy: There are several views concerning the origin of osmotic phenomena expressed as pressures (20). Osmosis has been conceived generally as involving pressures of solute and/or solvent. This approach is illogical and unnecessary and leads to confusion over the mechanics of this process. Although the action capacities, tending to cause water to move through a semipermeable membrane of an ideal osmometer, may be expressed in dimensions of $m L^{-1} t^{-2}$, these specific free energy quantities are merely measures of the escaping tendency of solvent water molecules. Based on the free energy concept, rather than actual exerted pressures of the component molecules, the process is presented with more clarity. In the study of osmotic "solute" specific free energy, the essential feature is that the free energy of the solvent molecules is less in a solution than in the pure liquid at constant temperature and pressure; in other words, the transfer of water through an interposed semipermeable membrane from its pure (standard) state to that in a solution will result in a decrease of free energy. Such a flow will, therefore, always tend to occur whenever solvent and solution are brought together. Where they are separated by a semipermeable membrane, the water must flow into the solution until equilibrium is attained by the building up of a hydrostatic specific free energy, or its equivalent pressure, within the solution phase, ideally equal to the osmotic

"solute" specific free energy, and related to the concentration of solute in the solution, in accordance with the van't Hoff equation (see equations 3, 4). The existence of an osmotic "solute" specific free energy is the inevitable result of the introduction of a semi-permeable membrane between a pure solvent and a solution, or between two solutions of dissimilar solute concentration, on account of the difference of the free energy of the solvent molecules in the two phases (compare the sections herein entitled "The Free Energy Concept", "Osmotic 'Solute' Specific Free Energy Defined", and "On the Origin of Osmotic 'Solute' Specific Free Energy" with the section entitled "The Free Energy of a Constituent Solute in a Solution", in Part II). See footnote 3.

Deviations in the Plant, from the Ideal Osmotic System:

I. Semipermeable membrane.

a. The thickness is finite.

1. Time is finite.

2. Semipermeability may be due to the restricted passage of the component (solute) not free to move through the limiting surface, through a capillary of, or through solution in, the bounding layer.

b. The membrane is never strictly semipermeable.

1. A metabolic specific free energy may be adapted to move unilaterally the component (solute) heretofore regarded as incapable of free passage through the semipermeable membrane through this differentially permeable boundary with or against (metabolic accumulation of solute) the direction in which its concentration decreases.

II. For each phase the change in volume is finite.

a. Time is finite.

b. After a finite time the system no longer involves pure components A (solvent, water) and B (solute), but rather component A (water) in phase e; and in phase i, a solution of A (water) and B (solute). (See Figure 1.) Further, since the membrane is not strictly semipermeable, the system may subsequently involve two solutions of the components, in phases e and i.

c. In the ideal aqueous osmotic system there should be no change in volume due to evaporation.

- III. Temperature changes are finite. However, in this treatise this factor is assumed to be non-variant.
- IV. Thermodynamic equilibrium, within the osmotic system as a whole, may be approached, but is probably never attained.
- V. The state of the system is not determined solely by the temperature, pressure and composition. Other independent variables, for example, gravitational and electrical fields, and surface effects, may be involved.

In the osmotic system of the plant, as indicated hereinbefore, the component capable of relatively free passage through the semi-permeable membrane is the solvent water. The component relatively incapable of free passage is the solute arising within the plant through metabolism or supplied to the organism from without. In the imperfect osmotic system of the plant, we have to deal not with two pure components, but with aqueous solutions, or colloidal systems, in the two membrane-separated phases.

Comparison Between Two Alternative Aspects of the Fundamental Equation for Water Movement in an Osmometer: There are two alternative methods of viewing the osmotic relations of plants, both based upon the fundamental physico-chemical principles of osmosis. The first method is useful only in a purely mathematical analysis of the system. This scheme has been developed through the cumulative efforts of a number of investigators, most recently advanced by Meyer and others (43-45). The second, alternative method, is useful in a purely mathematical analysis of the system and also serves to present, in a simple manner, the osmotic relations in graphic form, extending the diagrams of Thoday and Höfler (59, 27).

Consider the process in which water moves in an osmometer from a point external to the interposed membrane, to a point internally, *i.e.*, H_2O (external) $\xrightarrow{\text{influx}}$ H_2O (internal) (see 18, pp. 100-104). The measure of the tendency for water to move is given by the difference between the free energy of water in the internal phase and that in the external phase of the osmometer, $(\bar{f}_i - \bar{f}_e)$. If this difference is negative in sign, water will tend to move inward, as written. Thus, if it is desired to know whether or not water will tend to move across the membrane, the quantity $(\bar{f}_i - \bar{f}_e)$ must be determined.

In practice the free energies themselves are not determined, but the difference between the free energy in a given state and the free energy in a reference or standard state. For water the standard state is customarily chosen as that of pure water under standard conditions. If by thermodynamic methods the quantities $(\bar{f}_i - f^\circ)$ and $(\bar{f}_e - f^\circ)$ are determined, then the difference between these quantities is $(\bar{f}_i - \bar{f}_e)$.

As will become clear there is an entirely equivalent method of measuring the tendency for the above process to occur. This method involves the measurement of the osmotic specific free energies (F) in each phase, distinguishing between those constituent influences which tend to decrease the free energy of the component water in the given state with respect to that in the reference state $(-\Delta f)$, and those which correspondingly tend to increase the free energy (Δf) . The presence of solute or a hydrophilic imbibant in an aqueous medium will tend to lower the free energy of the solvent water. A hydrostatic pressure, or turgor in an enclosed phase, will raise the free energy above that in the reference state. This physico-chemical analysis of the osmotic specific free energies involved in the system will be presented by means of equations which apply only for dilute solutions and low pressures.

The osmotic "solute" specific free energy is defined as equal, both dimensionally and mathematically, to the increase in pressure on a solution necessary to make the free energy of the solvent in the given state, the same as that of the pure solvent. For dilute aqueous solutions, the osmotic "solute" specific free energy (F_s) is given by the equation:

$$F_s = p - p^\circ = \frac{-(\bar{f} - f^\circ)}{v^\circ} \quad (5)$$

where \bar{f} is the free energy of water in the solution; f° is the free energy of water in the pure state; v° is the molal volume of pure water; p° is the pressure on the water in the pure state; and p is the pressure necessary to make \bar{f} equal to f° .

Since the molal volume of pure water is a constant, it is evident from equation 5 that the osmotic "solute" specific free energy (F_s) is a measure of the quantity $(\bar{f} - f^\circ)$. In the absence of other osmotic influences on the system, it follows that

$$F_{s_i} - F_{s_e} = \frac{-(\bar{f}_i - \bar{f}_e)}{v^\circ} \quad (6)$$

where F_{s_i} and F_{s_e} are the antiphasic osmotic specific free energies related to the presence of internal solute and external solute, respectively. As previously stated, $(\bar{f}_i - \bar{f}_e)$ is a measure of the tendency for water to move across the membrane. From equation 6 it is apparent that the quantity $(F_{s_i} - F_{s_e})$ is also a measure of the tendency for water to move. A positive value for $(F_{s_i} - F_{s_e})$ indicates a tendency for water to move inward, *i.e.*, an influx of water.

The above method may be applied to cases where either or both of the phases of the osmometer may be subjected to other osmotic influences, isothermally. The relationships apply only for dilute solutions and low pressures. An increase in pressure on a phase of the system ordinarily is accompanied by an increase in the free energy of water. For water the change in free energy due to an imposed pressure is proportional to the change in pressure, *i.e.*,

$$F_{(\Delta f)} = -(p - p^\circ) = \frac{(\bar{f} - f^\circ)}{v^\circ}. \text{ On the other hand, the change in free}$$

energy of water due to the presence of dissolved material is proportional to the negative of the osmotic "solute" specific free energy,

$$\text{i.e., } F_{(-\Delta f)} = (p - p^\circ) = \frac{-(\bar{f} - f^\circ)}{v^\circ}. \text{ The two equations, therefore,}$$

have the same form, but opposite signs. If the specific free energies, equal to the imposed pressures necessary to make \bar{f} equal to f° , are designated by the symbol $F_{(-\Delta f)}$ for constituent influences lowering the free energy of the solvent water, and by the symbol $F_{(\Delta f)}$ for constituent influences raising the free energy, it follows that

$$(\sum F_{(-\Delta f)} - \sum F_{(\Delta f)}) = \frac{-(\bar{f} - f^\circ)}{v^\circ} \quad (7, \text{ see equation 2})$$

and for the two phases, that

$$((\sum F_{(-\Delta f)}) - (\sum F_{(\Delta f)}))_i - ((\sum F_{(-\Delta f)}) - (\sum F_{(\Delta f)}))_e = \frac{-(\bar{f}_i - \bar{f}_e)}{v^\circ} \\ = \text{DPDD} = \text{NIF} \quad (8)$$

Thus the expression, $((\sum F_{(-\Delta f)}) - (\sum F_{(\Delta f)}))_i - ((\sum F_{(-\Delta f)}) - (\sum F_{(\Delta f)}))_e$, is also a measure of the tendency for water to move. A positive value for this quantitative difference indicates a tendency for water to move inward, *i.e.*, an influx of water. Two typical examples of the measurement of the tendency for water to move may be taken from equations 12 and 36, to be discussed later. For

example, the tendency for water to move inward = $((Fs_i) - (Fh_i)) - (Fs_e)$. (9a)

Again, the tendency for water to move inward = $((Fs_i) - (Fh_i)) - ((Fs_e + Fnm) - (Fm))$. (9b)⁷

In the terminology of Meyer these tendencies for water to move inward are expressed in each case by the difference between the algebraic sum of the diffusion pressure deficits for water associated with internal factors, and the algebraic sum of the diffusion pressure deficits for water associated with external factors, where the difference is represented by the equations:

$$DPDD = (\sum DPD)_i - (\sum DPD)_e \text{ and} \quad (10)$$

$$DPDD = (OP + (-TP))_i - (OP)_e; \text{ or} \quad (10a\text{---see equations } 9a \text{ and } 12)$$

$$= (OP + \dots + (-TP))_i - (OP + \dots + \dots)_e. \quad (10b\text{---see equations } 9b \text{ and } 36)$$

In order to express these specific free energies in equations and in graphic form, where the tendencies for water flow may be followed in relation to the changes in the external and internal specific free energies and the changes in the internal volume (the latter heretofore considered constant), an alternative scheme was developed. Here, the difference between the algebraic sum of the action capacities tending to cause water to move inward and the algebraic sum of those tending to cause water to move outward is a measure of the net tendency for water to move inward across the membrane. The osmotic specific free energies, regrouped in the equation of net tendency for water flow, are expressed in terms of the changes in the actual osmotic specific free energy of the water molecules, due to existing action capacities related to the constituent influences in an osmometer phase. In other words, the constituent

⁷ It may be observed that the metabolic specific free energy factor tending to cause the component (water here) to move inward in the system is designated as an influence tending to increase the free energy of the component in favor of the external phase. The over-all movement of the component against the direction in which its concentration decreases, is in the direction opposite to that in which a flow "normally" would occur and is therefore associated with an apparent increase in free energy between the two phases. The law of tendency toward flow with the direction in which the specific free energy of the component decreases is maintained by asserting a complementary decrease of free energy within the cytoplasm, more than sufficient to compensate for the increase in question (35, pp. 120, 121). Oxidative catabolism would be required in this way for metabolic accumulation of water.

specific free energies of the system, related as expressed in equation 8, are regrouped to accord with the net influx specific free energy equation 4 (see p. 9). Thus, the equation

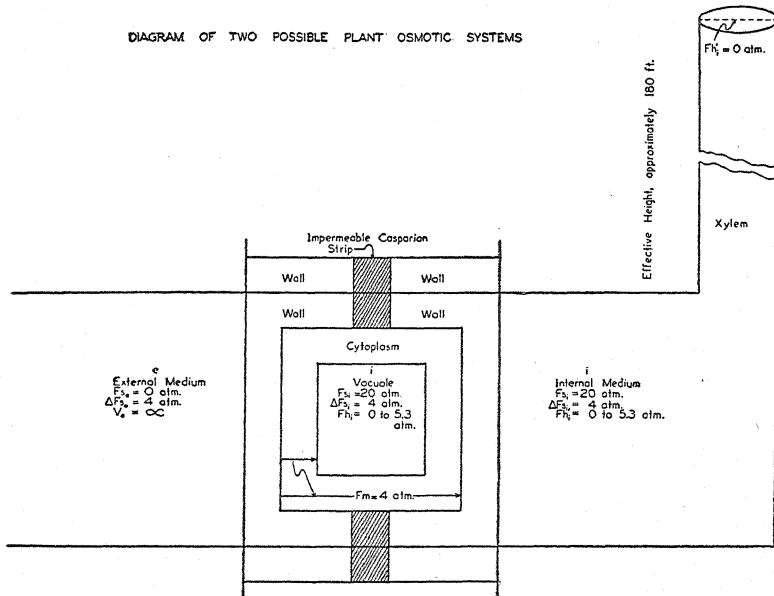
$$\text{NIF} = \Sigma \text{IF} - \Sigma \text{EF} \quad (4)$$

is equivalent to

$$\text{NIF} = ((\Sigma F_{(-\Delta f)})_i + (\Sigma F_{(\Delta f)})_e) - ((\Sigma F_{(-\Delta f)})_e + (\Sigma F_{(\Delta f)})_i). \quad (4a)$$

MOVEMENT OF WATER IN THE PLANT: THE ALTERNATIVE GRAPHIC SCHEME

In the water relations of the plant⁸ (34, 37), two reference



In the initial state the volume of the external medium is assumed to be infinite, that of the internal medium, relatively small. Hydrostatic specific free energies assumed to be zero, initially.

Fig. 2. Diagram of two possible plant osmotic systems. The two systems represented are, first, the simplified, integrated osmotic system, external medium (water, nutrient solution, or soil medium)—membrane (endodermal cytoplasm or protoplasm of a comparably structured cell series)—internal medium (xylem solution, *etc.*); and second, a reference cell, external medium (nutrient solution or intercellular solution)—membrane (cytoplasm)—internal medium (vacuolar solution).

⁸Water movement by mass flow along continuous water columns (uninterrupted by differentially permeable septa) from root to shoot, similarly governed by the free energy difference of the solvent molecules, involving evaporation and cohesion, will not be discussed in detail (see 15, 28, 34, 45, 60; compare 51, 53).

TABLE I

SPECIFIC FREE ENERGIES INVOLVED IN THE MOVEMENT OF WATER INTO PLANTS UNDER NATURAL ENVIRONMENTAL CONDITIONS*

Net Influx Specific Free Energy = Σ Influx Specific Free Energies - Σ Efflux Specific Free Energies $NIF = \Sigma IF - \Sigma EF$

<i>Water Influx Specific Free Energies (IF)</i>	<i>Water Efflux Specific Free Energies (EF)</i>
<p>A. Osmotic "solute" specific free energy (antiphasically effective), related to the presence of internal solute[†], arising through solute (electrolyte and/or non-electrolyte) influx (by simple diffusion, Donnan diffusion, exchange adsorption or metabolic accumulation) into the medium bathing the inner surface of the cell (endoplasm), or of the endodermis or comparably structured cell series. $F_{s_i} = IF$.</p> <p>B. "Negative" hydrostatic specific free energy resulting from hydrostatic tension (negative pressure), internal, $-F_{h_i} = \Delta IF$. Intrinsic hydrostatic specific free energy plus extrinsic hydrostatic specific free energy, i.e., $F_{h_i} = F_{h_i'} + F_{h_i''}$.</p> <ol style="list-style-type: none"> 1. Open inner phase: here $F_{h_i'}$ is positive and $F_{h_i''}$ is negative; that is, a partial solution tension exists exceeding $F_{h_i'}$ numerically. 2. Inclosed inner phase: here $F_{h_i'}$ is zero or negative (resulting from intracellular tension, membrane ("wall") tension). $F_{h_i''}$ is zero or negative, usually varying directly as $F_{h_i'}$ (resulting from intercellular tension). <p>C. Hydrostatic specific free energy resulting from hydrostatic pressure, external. $F_{h_e} = \Delta IF$ ($F_{h_e} = F_{h_e'} + F_{h_e''}$).</p> <p>D. Metabolic specific free energy, inwardly directed; arising within the living cytoplasm, $F_m = \Delta IF$.</p> <p>E. Non-metabolic specific free energy, inwardly directed. $F_{nm} = \Delta IF$.</p> <ol style="list-style-type: none"> 1. Internal imbiban (Fnm_i), antiphasically effective. 	<p>A. Hydrostatic specific free energy resulting from hydrostatic pressure, internal. $F_{h_i} = EF$. Intrinsic hydrostatic specific free energy plus extrinsic hydrostatic specific free energy; i.e., $F_{h_i} = F_{h_i'} + F_{h_i''}$.</p> <ol style="list-style-type: none"> 1. Open inner phase: Here $F_{h_i'}$ is positive and $F_{h_i''}$ is either zero or negative; that is, a partial solution tension less numerically than $F_{h_i'}$ may or may not exist. 2. Inclosed inner phase (resulting from hydrostatic pressure): Here $F_{h_i'}$ is positive or zero (resulting from intracellular pressure, membrane ("wall") pressure). $F_{h_i''}$ is positive or zero, usually varying directly as $F_{h_i'}$ (resulting from intercellular pressure). <p>B. Osmotic solute specific free energy (antiphasically effective) related to the presence of external solute. $F_{s_e} = \Delta EF$.</p> <p>C. Metabolic specific free energy, outwardly directed. $F_m = \Delta EF$.</p> <p>D. Non-metabolic specific free energy, outwardly directed. $F_{nm} = \Delta EF$.</p> <ol style="list-style-type: none"> 1. External imbiban (Fnm_e), antiphasically effective.

* Unless otherwise specified (see sections on effect of suction and miscellaneous effects), all pressures are above the reference atmospheric pressure,

systems will be examined (see Figure 2): first, the simplified, integrated osmotic system (6, 12, 52), external solution—membrane (endodermal cytoplasm or protoplasm of a comparably structured cell series)—internal solution; and second, a reference cell, external solution—membrane (cytoplasm)—internal solution (vacuole). Although probably not strictly comparable, these will be discussed as similar systems. Modifications of similarity will be presented. The dynamics of osmotic water movement governed in any case by the over-all free energy difference of the water molecules in the system are expressed, in a summarized form, by the relations in Table I. The net free energy of the water molecules tending to cause its movement into the plant osmometer, equals the free energy of the water molecules in the external phase diminished by the free energy of the water molecules in the internal phase⁹. This treatise presents the constituent osmotic specific free energies which are measures of the free energies of the water molecules in the two phases, regrouped into their categories as influx or efflux specific free energies.

The Fundamental Equation (see 64, p. 437): From equation 3b it may be seen that the greater the concentration of solute in a solution, the greater is the antiphasic osmotic "solute" specific free energy. Since the free energy of the water molecules is lowered by this means in the internal phase of an osmometer, the efflux specific free energy for water is reduced and the influx specific free energy for water is proportionately increased. Water will enter the osmometer due to the higher solute concentration of the internal

which is real and equal throughout the systems described. Here, the use of a reference pressure and the term net influx specific free energy are not open to the criticism posed by Crafts (11, p. 387) in connection with other modes of presentation of osmotic relations.

† The externally effective osmotic "solute" specific free energy is related to the antiphasic solute concentration, in the internal phase (see equations 3 and 3a). The internally effective osmotic "solute" specific free energy is similarly related to the solute concentration in the external phase of the osmometer, bathing the ectoplasm of the reference cell or outer surface of the endodermis or cell series.

⁹ The external or internal phases of either of the reference osmometers may be considered as individual units (see footnote 10) with respect to the free energy of the water. At equilibrium, the free energy of the water is constant throughout a phase, with relation to a reference level in space, regardless of its shape or size. At a lower position in space within the phase, the free energy is diminished on account of the influence of gravity, but it is increased by the intrinsic hydrostatic pressure (due to the mass of the column alone); these two influences just offset one another (35, pp. 242, 243).

phase¹⁰. If in an open system a pressure head of fluid develops, due to the entry of water, the influx specific free energy, related to the solute concentration difference alone, will be diminished by a counter specific free energy within the osmometer. This latter specific free energy results from the hydrostatic pressure due to the weight of the heightened column of the inner solution. This complementary specific free energy within the osmometer is termed the intrinsic hydrostatic specific free energy (F_{hi}'). Water will enter until this specific free energy in the internal solution counterbalances the osmotic specific free energy in the external phase (F_{si}), related to the presence of internal solute. In an enclosed osmotic system, exemplified by an isolated reference cell, a counter pressure on the membrane is also produced. Instead of a hydrostatic pressure due to the weight of a column of liquid, an outwardly directed hydrostatic pressure is involved, counteracted by the restricted expansion imposed by the elastic membrane encircling the inner phase. As in the open inner phase system, water will enter the inclosed phase until the internal hydrostatic specific free energy, resulting from the internal hydrostatic pressure on the membrane, counterbalances the osmotic specific free energy related to the presence of internal solute.

On osmotic entry of water, to satisfy an existing free energy difference, the net influx specific free energy is diminished. The osmotic specific free energy related to internal solute is decreased by dilution of the internal medium, and the hydrostatic specific free energy is concurrently increased. The relative changes in the latter quantities will depend upon the extensibility of the inner phase. The lower the extensibility of the inner phase, the smaller will be the water influx accompanied by a relatively smaller decrease in the osmotic "solute" specific free energy and a relatively larger increase in the intrinsic hydrostatic specific free energy.

Ideally, the relations between changes in the relative volume of the inner phase of an osmometer and the hydrostatic specific free

¹⁰ When the solute concentration of the medium is not uniform (as in the xylem, even at thermodynamic equilibrium (35, pp. 243, 244), for example), an osmotic "solute" specific free energy (within the opposite phase) related to the weighted average concentration of the solution within the phase, is employed. Localized changes of solute concentration within a phase may modify the osmotic "solute" specific free energy relations so as to alter the rate of water flow, or even its direction, through the membrane. Such thermodynamic non-equilibria would probably be rapidly adjusted, approaching those under discussion, unless the differential is continuously maintained.

energy are linear. However, single or multiple deviations from linearity occur in reality. Thus, accentuated successive changes in the thickness of the membrane (causing modifications of its modulus of elasticity), due to internal changes of volume, may lead to a "curvilinear" relation in which the second derivative of the hydrostatic specific free energy with respect to volume is negative. Contrariwise, pronounced successive changes in restriction to modification of the internal volume may lead to a "curvilinear" relation in which the second derivative of the hydrostatic specific free energy with respect to volume is positive.

In the initial state of a simplified osmotic system, as represented by the integrated endodermal system in Figure 2, in which the numbers employed in atmospheres are of magnitudes similar to those found experimentally, the fundamental equation for water movement might require, for example, the following values:

$$\text{NIF} = \text{Fs}_1 - \text{Fh}_1' \quad (11)^{11}$$

$$20.0 = 20.0 - 0 \text{ (see Figure 3)} \quad (11a)$$

If a difference in free energy of water molecules is partially satisfied through influx of water (endosmosis) with changes in specific free energies and internal volume of the system¹², equation 11a may become, for example, after a finite interval of time,

$$13.0 = 18.3 - 5.3 \quad (11b)$$

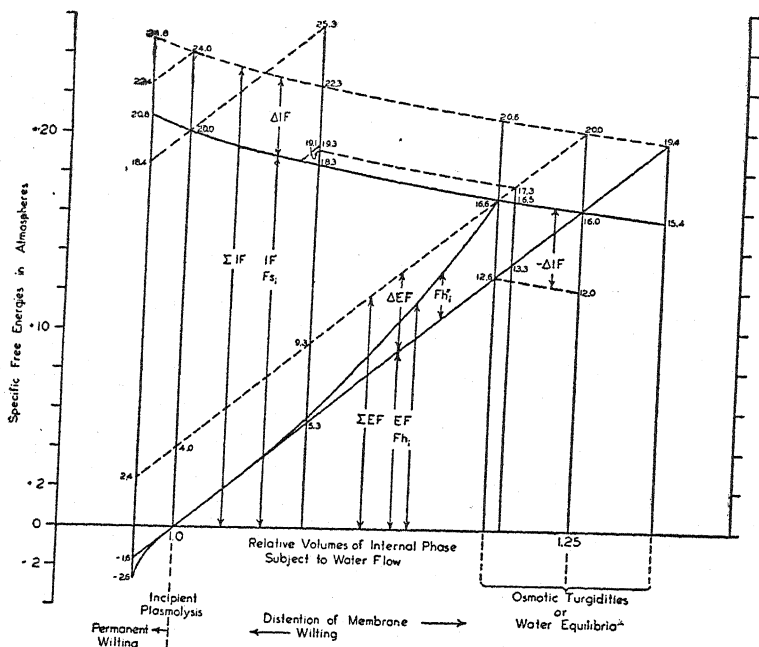
(in Figure 3, $\text{NIF} = 18.3 - 5.3 = 13.0$) and at water equilibrium

$$0 = 16.0 - 16.0 \quad (11c)$$

It is a common practice by investigators to insert manometers into the hydrostatic system of intact plants, especially species which grow to relatively great heights. It may be noted from these premises and those to follow that these instruments record the hydrostatic pressure (Ph_1), the resultant between the intrinsic hydrostatic pressure and any possible extrinsic hydrostatic pressure or tension, existing at that time and level within the internal phase of the reference osmometer. The related hydrostatic specific free energy (Fh_1) is thereby evaluated.

¹¹ Similar here to the fundamental equation of Meyer, where the resultant $\text{DPD} = \text{OP} - \text{TP}$. Also, see 6, 27, 40, 52, 57, 59, 61, 64.

¹² Diminution of the internal volume of the osmometer by loss of water must have the effect of increasing the internal concentration of substances to which the membrane is relatively impermeable. By mass action, reactions of which these substances are components will be accelerated, and increase in volume by influx of water will retard such reactions.



WATER RELATIONS OF A PLANT OSMOMETER

The Net Influx Specific Free Energy is equal to the sum of the Influx Specific Free Energies diminished by the sum of the Efflux Specific Free Energies ($\text{NIF} = \Sigma \text{IF} - \Sigma \text{EF}$). NIF is expressed by the difference between the scalars, ΣIF and ΣEF , at any particular volume in the Specific Free Energy: Volume diagram.

FIG. 3. Water relations of a plant osmometer: the general scheme. The hydrostatic specific free energies in relation to changes in volume of the internal phase of the osmometer may be represented, within the elastic limits of the membrane, by either an approximately linear or a sigmoid curvilinear function, each passing through the origin of zero specific free energy and unit relative volume. Values for the corresponding osmotic "solute" specific free energies (antiphasically effective) related to the presence of solute in the internal medium, represented under isothermal conditions, form a hyperbolic function within the limits of applicability of the van't Hoff equation. The slopes of the hydrostatic specific free energy, and osmotic specific free energy (the latter related to the presence of solute in the internal medium) curves (EF and IF) will be governed by the elasticity of the membrane and the nature of the constituent factors. For simplicity, supplementary specific free energy increments or decrements are considered as constants; as such, they are diagrammatically represented by curves paralleling the fundamental osmotic values of IF and EF. Likewise, the two osmotic systems exemplified are discussed with values of specific free energy, or volume, of similar magnitude. Here, where necessary, it is assumed that the isothermal temperature was 25° C. Because the specific free energy values were somewhat arbitrarily selected, although approximating experimentally observed quantities, and since data are lacking to justify the application of an osmotic "solute" specific free energy correction other than unity, it is assumed here that this coefficient is equal to one (see footnote 6).

Effect of External Solute: If the free energy of the water molecules is lowered by the presence of solute in the external phase of the osmometer the efflux specific free energy for water is increased, and, therefore, other osmotic quantities remaining constant, the net influx specific free energy is reduced. Entry of water, to satisfy the net specific free energy tending to cause water to move inward, yields figures at equilibrium differing from those in the fundamental equation. The volume increase in the internal phase is lessened, and therefore the equilibrium value for the osmotic specific free energy related to internal solute is greater. The corresponding efflux specific free energy is higher. Here, this efflux specific free energy comprises two quantities, the osmotic "solute" specific free energy (F_{s_e}), related to the solute concentration of the external solution (assumed to be constant), and a diminished hydrostatic specific free energy.

If the system is bathed externally by a solution with a solute concentration related to an osmotic solute specific free energy of four atmospheres, for example (in place of the initial component water), equation 11a takes the form

$$NIF = F_{s_i} - (F_{h_i}' + F_{s_e}), \quad (12)$$

where F_{s_e} is a positive ΔEF ,

$$16.0 = 20.0 - (0 + 4.0). \quad (12a)$$

If free energy gradients are partially satisfied through influx of water, equation 12a may become

$$9.0 = 18.3 - (5.3 + 4.0) \quad (12b)$$

(in Figure 3, $NIF = 18.3 - 9.3 = 9.0$) and at water equilibrium

$$0 = 16.6 - (12.6 + 4.0) \quad (12c)$$

Effect of Internal Solute: Solute Accumulation: Addition of solute to the internal phase of the osmometer, as represented by the fundamental equation, would increase the influx specific free energy for water by lowering the free energy of the solvent internally. At water equilibrium a greater volume is attained than would be obtained with the relatively lower internal solute concentration. The corresponding efflux or hydrostatic specific free energy is accordingly increased.

If in the system at water equilibrium, further solute is added by any means¹³, to the solution internally, related to an osmotic "solute"

¹³ Influx of solute (electrolyte or non-electrolyte) through redistribution within the plant by diffusion, involving undifferentiated or differentiated cells or tissues, may ensue (52). In effect, this could occur also, for example, through hydrolysis or decondensation of solute already present in the internal phase.

specific free energy of four atmospheres, for example, equation 11c

$$\text{NIF} = F_{s_i} - F_{h_i'} \quad (11)$$

$$0 = 16.0 - 16.0 \quad (11c)$$

may become

$$\text{NIF} = (F_{s_i} + \Delta F_{s_i}) - F_{h_i'} \quad (13, \text{ see } 11)$$

$$4.0 = (16.0 + 4.0) - 16.0. \quad (13a)$$

If differences of free energy of water are satisfied, then at equilibrium

$$\text{NIF} = F_{s_i} - F_{h_i'} \quad (11)$$

$$0 = 19.4 - 19.4. \quad (13b)$$

Similarly, if to the system represented by equation 12c, in which

$$\text{NIF} = F_{s_i} - (F_{h_i'} + F_{s_e}) \quad (12)$$

$$0 = 16.6 - (12.6 + 4.0) \quad (12c)$$

further solute influx takes place, especially by metabolic accumulation from the external medium, equal to an internal increase of solute related to an osmotic "solute" specific free energy of four atmospheres, for example, the following relations arise in which

$$\text{NIF} = (F_{s_i} + \Delta F_{s_i}) - (F_{h_i'} + F_{s_e}) \quad (14, \text{ see equation } 12)$$

$$4.0 = (16.6 + 4.0) - (12.6 + 4.0) \quad (14a)$$

At water equilibrium, this becomes

$$\text{NIF} = F_{s_i} - (F_{h_i'} + F_{s_e}) \quad (12)$$

$$0 = 20.0 - (16.0 + 4.0) \quad (14b)$$

Effect of Internal Solute Depletion: The osmotic system at water equilibrium expressed by equation 11c (compare equation 16) may decrease with respect to its osmotic specific free energy related to the presence of internal solute, apart from a solute efflux to the external phase occasioned by possible injury, accompanied by water. Such a decrease in osmotic "solute" specific free energy might occur when either solute is translocated, without resupply from *e* (see Figure 2), from the inner phase of the reference cell (vacuole); or on loss of solute from the open osmometer system due to exudation; or due to a possible condensation of solute internally. The osmotic "solute" specific free energy related to the presence of internal solute would be decreased with a corresponding reduction in the net influx specific free energy, for water. If the system were at equilibrium with respect to water, a net specific free energy tending to cause water to move out of the osmotic system may become operative. In any case, as above, such a reduction in

osmotic specific free energy related to the presence of internal solute would lead to a new water equilibrium where the internal volume is decreased and the hydrostatic specific free energy lowered correspondingly. An internal solute depletion of this type equivalent to four atmospheres osmotic "solute" specific free energy, for example, would be represented by a negative change in the influx specific free energy ($-\Delta IF$).

Equation 11c

$$NIF = F_{S_i} - F_{h_i}' \quad (11)$$

$$0 = 16.0 - 16.0 \quad (11c)$$

would become

$$NIF = (F_{S_i} + \Delta F_{S_i}) - F_{h_i}' \quad (15)$$

$$-4.0 = (16.0 - 4.0) - 16.0 \quad (15a)$$

tending to cause an efflux of water.

At water equilibrium, provided this condition would be realized¹⁴ through an internal supply of water, the following relations would exist,

$$NIF = F_{S_i} - F_{h_i}'$$

$$0 = 12.6 - 12.6 \quad (15b)$$

Exudation (See paragraphs in section entitled "The Comprehensive Osmotic System of the Plant"): If the inner phase of the osmotic system is not inclosed, but rather, open, as is the xylem in the system external solution—endodermis—internal solution, a counter hydrostatic specific free energy arises resulting from the hydrostatic pressure set up due to the weight per unit cross section of the column of solution, as shown by equations 12a, 12b and 12c, whenever an inwardly decreasing specific free energy of water exists. If a net influx specific free energy is realized when the hydrostatic specific free energy, resulting from the hydrostatic pressure, is equivalent to the effective height of the inner phase (xylem)¹⁵, water will tend to exude from the upper surface as long

¹⁴ Compare section entitled "Effect of External Salinity on the Open Reference System".

¹⁵ Disregarding capillary rise, involving surface tension, etc. Capillarity is not specifically discussed as an influence leading to the flow of water in an osmometer system because this factor, once satisfied, remains relatively constant within limited time periods. Over longer intervals of time, it may vary in the plant, with growth; in the soil, with changes in the structure of the medium. Any effect of capillarity on the systems as analyzed will reduce the free energy of the water molecules within the osmometer phase concerned. If the inner phase is viewed as of significant capillary dimension, an influx specific free energy (ΔIF), resulting from a constituent internal intrinsic hydrostatic tension, will tend to cause water to move into the osmometer. Any rise of the solution within a capillary column above the hydrostatic reference level allowed by the osmotic action capacities discussed will, to this extent, increase the effective height of the inner phase.

as the required gradient of free energy of water is maintained, provided it be assumed that evaporation is negligible and the rate of water influx is greater than the water requirement of the tissues attendant on growth. Under these conditions the net influx specific free energy is termed the "specific free energy of exudation". Exudation from a decapitated plant is frequently termed "bleeding". When the flow of fluid occurs from the distal ends of xylem strands through hydathodes (tissue modifications for ease of fluid exit), the exudation is termed "guttation". For a particular system, other factors being constant, the rate of exudation is proportional to the magnitude of the net influx specific free energy (NIF). The absolute rate of water flow will equal the product of the permeability coefficient of the osmometer membrane and the net influx specific free energy gradient (Part II).

If an external solution of solute concentration equivalent to an osmotic "solute" specific free energy of 13.0 atmospheres is applied to the system expressed by equation 12a, the exudation, at an effective height equivalent to 5.3 atmospheres hydrostatic specific free energy, would be stopped and could be reversed if the osmotic specific free energy related to the presence of external solute exceeds 13.0 atmospheres.

Thus,

$$\text{NIF} = F_{s_i} - (F_{h_i'} + F_{s_o}) \quad (12)$$

$$16.0 = 20.0 - (0 + 4.0) \quad (12a)$$

tending to cause an influx of water with exudation; and

$$0 = 18.3 - (5.3 + 13.0) \quad (17)$$

water equilibrium; and

$$-1 = 18.3 - (5.3 + 14.0) \quad (18)$$

tending to cause an efflux of water.

Effect of a Metabolic Specific Free Energy: It has been suggested by some investigators (4, 5, 40, 42, 46, 49, 50, 66, 67; compare 13, 16, 54 and 58, 38 and 39) that under certain conditions water may move against the direction in which its concentration decreases, into the plant or plant cell. Such a flow would require direct utilization of respiratory energy¹⁶. This specific free energy, if such is normally effective in living plants, may be termed a metabolic specific

¹⁶ An adequate supply of metabolite is essential to this rôle. Labile carbohydrate could serve here in two ways: directly as an osmotic solute, indirectly as a source of osmotic energy through metabolism. Obviously, its effectiveness, by these two means of water flow, is interconnected.

free energy, since the process at any time would be unidirectional and maintained through metabolism of the living cell or organism. Such a metabolic specific free energy might, for example, be related to an electrical potential difference across the membrane or between the external and internal phases, produced directly through solute migration or other means (5, 6; compare 38, 39, 49), or a temperature differential between the external and internal phases. It should be noted that such a metabolic specific free energy may also lead to unilateral movement of water with the direction in which its concentration decreases. In the latter case the metabolic specific free energy is merely serving to increase the rate of flow toward a water equilibrium demanded by an independently established net influx specific free energy. If a metabolic specific free energy is normally effective in tending to cause water to move against the direction in which its concentration decreases, it remains to be shown how the living organism governs its metabolic processes to direct the diffusion of solute and solvent through or across the cytoplasmic membrane at the same or separate intervals of time or space against the direction in which the concentrations decrease. Such a concept is theoretically possible, if water is considered (apart from its greater proportion in solution where it is regarded as solvent) like any other typical chemical species, discussed under the heading of solute accumulation.

Application of a metabolic influx specific free energy, associated with an apparent external increase of the free energy of the water molecules, would increase the net influx specific free energy and the rate of water flow. Entry of water to equilibrium would lead to an increase in the volume of the inner phase with a reduction in solute concentration of the internal solution, *i.e.*, a reduction of the osmotic specific free energy related to the presence of internal solute; and an increase of the corresponding specific free energy, resulting from an increase of internal hydrostatic pressure on the membrane. Under conditions of exudations, the specific free energy of exudation would be increased. In order to counteract the possible effects of such a metabolic specific free energy, application of an opposing specific free energy, or pressure of equivalent magnitude, would have to be applied. (Note the specific free energy required for plasmolysis and interruption of exudation.)

If the plant is able to tend to cause water to move against the

direction in which its concentration decreases, as discussed, then equations 11c and 12a, 16 and 17 would be modified quantitatively. A metabolic influx of water might lead to values represented by the following:

$$\text{NIF} = (\text{Fs}_i + \text{Fm}) - \text{Fh}_i'$$

or $\text{NIF} = (\text{Fs}_i + \text{Fm}) - (\text{Fh}_i' + \text{Fs}_e)$, in which if Fm represents a metabolic influx specific free energy of four atmospheres, for example, then

$$\text{NIF} = \text{Fs}_i - \text{Fh}_i' \quad (11)$$

$$0 = 16.0 - 16.0 \quad (11c)$$

becomes

$$\text{NIF} = (\text{Fs}_i + \text{Fm}) - \text{Fh}_i' \quad (19)$$

$$4.0 = (16.0 + 4.0) - 16.0 \quad (19a)$$

and at water equilibrium

$$0 = (15.4 + 4.0) - 19.4 \quad (19b)$$

Likewise, equations 12a, 16, and 17

$$\text{NIF} = \text{Fs}_i - (\text{Fh}_i' + \text{Fs}_e) \quad (12)$$

$$16.0 = 20.0 - (0 + 4.0) \quad (12a)$$

$$9.0 = 18.3 - (5.3 + 4.0) \quad (16)$$

$$0 = 18.3 - (5.3 + 13.0) \quad (17)$$

will become respectively

$$\text{NIF} = (\text{Fs}_i + \text{Fm}) - (\text{Fh}_i' + \text{Fs}_e) \quad (20)$$

$$20.0 = (20.0 + 4.0) - (0 + 4.0) \quad (20a)$$

$$13.0 = (18.3 + 4.0) - (5.3 + 4.0) \quad (21)$$

tending to cause an influx of water with exudation, and

$$0 = (18.3 + 4.0) - (5.3 + 17.0) \quad (22)$$

water equilibrium.

Obviously, under these conditions of a possible metabolic influx specific free energy of four atmospheres, an external solution of a concentration related to an osmotic "solute" specific free energy of 17.0 atmospheres, an increase equal to the metabolic specific free energy, would then be required to prevent exudation.

Effect of a Non-metabolic Specific Free Energy: If an imbibant, or action capacity similar to that associated with imbibition, is present in the external medium, the free energy of the water molecules is lowered externally. An osmotic, non-metabolic, efflux specific free energy related to an external imbibant (Fnm_e , a ΔEF) may arise also from this source, counteracting specific free energy tending to cause water to move into the plant (see later section en-

titled "Soil Effects"). Conversely, the presence of an imbibant in the internal phase of a plant osmometer, lowering the free energy of the water molecules therein, would act as a non-metabolic influx specific free energy (F_{nm_i} , a ΔIF), tending to cause water to move through the membrane into the inner phase (28, 31, 58). Other non-metabolic specific free energy influences may include adsorption and capillarity. The latter, however, might be more logically conceived as a negative, constituent part of the intrinsic hydrostatic specific free energy within its phase.

Effect of Suction, Applied Internally: Although the effect of suction applied internally is not a natural situation, it is discussed here as an introduction to the effects of the natural internal tension caused by evaporation, as from the shoot of an intact plant. The over-all water movement may be modified by application of an extraneous internal or external pressure. Internally applied suction has a maximum pressure limit equivalent to atmospheric pressure. This suction is virtually an extraneously imposed external pressure equal to the reduction of the internal atmospheric pressure¹⁷. Application of suction internally, or an extrinsic hydrostatic pressure externally, increases the net influx specific free energy for water with its accompanying effects. Thus, if the osmometer height were unlimited, the influx of water to satisfy the increased net influx specific free energy would cause a decrease of the osmotic specific free energy related to the presence of internal solute (a decrease in solute concentration of the internal solution) and an increased internal hydrostatic pressure.

Subjecting the inner phase to such a reduced pressure of 0.8 atmosphere, for example, equation 16,

$$NIF = F_{s_i} - (F_{h_i}' + F_{s_e}) \quad (12)$$

$$9.0 = 18.3 - (5.3 + 4.0) \quad (16)$$

tending to cause exudation, might become

$$NIF = (F_{s_i} + \Delta IF) - (F_{h_i}' + F_{s_e}), \text{ or}$$

$$NIF = (F_{s_i} + F_{h_e}'') - (F_{h_i}' + F_{s_e}) \quad (23)$$

where ΔIF or F_{h_e}'' is equal to the atmospheric pressure difference existing on the two sides of the membrane

$$9.8 = (18.3 + 0.8) - (5.3 + 4.0) \quad (23a)$$

tending to cause an extraneous additional exudation.

¹⁷ See footnote *, table I. The same results are obtained whether the specific free energy change is considered as a ΔIF or as a $-\Delta EF$.

The rate of exudation is increased in proportion to the ratio NIF (equation 23a)/NIF (equation 16) = 9.8/9.0, provided other factors concerned in the rate of flow remain constant (Part II). If the osmometer height were unlimited and the water flow unrestricted, at water equilibrium equation 23a would assume the values

$$0 = (16.5 + 0.8) - (13.3 + 4.0) \quad (23b)$$

Effect of Evaporation: Internal Tension: Under normal conditions in the plant, evaporation of water from the inner phase^{17a} of the simplified integrated osmometer may set up an internal partial solution tension when the rate of supply of water furnished by the net influx specific free energy, exclusive of any such tension, is less than the rate of evaporation. Here, instead of the limited increase in influx specific free energy accomplished through internally applied suction (see discussion in preceding section), the excess water loss by evaporation causes an unrestricted real tension accompanied by a diminished internal hydrostatic specific free energy resulting from a diminished internal hydrostatic pressure, and by an increase in the osmotic specific free energy related to the presence of internal solute (compare equations 16, 23a and 23b above with 16 and 24 below). Thus, after a finite interval of time, equation 16

$$\text{NIF} = F_{s_1} - (F_{h_1} + F_{s_e}) \quad (12)$$

$$9.0 = 18.3 - (5.3 + 4.0) \quad (16)$$

may become

$$16.0 = 20.0 - (0 + 4.0) \quad (24)$$

In this case the internal hydrostatic specific free energy may virtually be reduced to zero by evaporation. In the plant, however, the intrinsic hydrostatic specific free energy, or pressure, related to the weight per unit cross section of the inner phase of the osmometer, is seldom materially reduced, but, on the other hand, is often maintained at or near its maximum value. When the rate of evaporation from the inner phase of the plant osmometer plus water required by growth exceeds the rate of water supply across the semipermeable membrane, the internal hydrostatic specific free energy (an efflux specific free energy) is the net result of two

^{17a} As evaporation occurs from a fluid column, the liquid-gas interface will recede. However, when this recession of the boundary is restricted by a perforate septum (e.g., cellulose), evaporation may give rise to a partial solution tension within the liquid phase, when the rate of supply of water furnished by a net influx specific free energy, exclusive of any such tension, is less than the rate of evaporation (28).

scalars, the intrinsic hydrostatic specific free energy and the extrinsic hydrostatic specific free energy decrement, *i.e.*, an intrinsic hydrostatic specific free energy resulting from an intrinsic hydrostatic pressure (due to the weight per unit cross section of the liquid column) diminished by an extrinsic hydrostatic specific free energy decrement, resulting from an internal partial solution tension (due to an internal negative pressure of internal extraphasic origin). In equation 24, therefore, the hydrostatic specific free energy, Fh_i , is a net quantity equal to $5.3 - 5.3 = 0$, *i.e.*, $Fh_i' + (-Fh_i'')$. If the evaporation rate were to continue to increase beyond the conditions imposed by equation 24, a hydrostatic tension would arise actuating an influx specific free energy tending to cause water to move into the inner phase of the osmometer. The following relations may then be obtained:

$$NIF = (Fs_i + (-Fh_i)) - (Fs_o) \quad (25)$$

$$18.4 = (20.8 + 1.6) - (4.0) \quad (25a)$$

Since the height of the water column has not materially changed, the intrinsic hydrostatic specific free energy remains at approximately 5.3 atmospheres. The internal hydrostatic "negative" specific free energy, $(-Fh_i) = 1.6$, antiphasically effective, is then the result of the difference $5.3 - 6.9$, *i.e.*, $Fh_i' + (-Fh_i'')$. In Figure 3, $(-Fh_i) = 1.6$ is obtained from the coordinates $(0 - (-1.6))$, and $Fs_o = 4.0$ from $(2.4 - (-1.6))$.

In equation 16 the net influx specific free energy was hypothetically moving water to an effective height commensurate with 5.3 atmospheres hydrostatic specific free energy, as an exudate. The extrinsic hydrostatic specific free energy decrement, resulting from an internal partial solution tension due to evaporation of water from the shoot, increases the net specific free energy tending to cause water to move inward through the system. However, the exudation of fluid from the surface of the inner phase apparently soon ceases when evaporation occurs. This relation will be obtained when the rate of evaporation plus water required by growth exceeds the rate of influx of water; then $-Fh_i'' > 0$. The specific free energy of exudation is equal to the net influx specific free energy in the absence of an extrinsic hydrostatic specific free energy decrement, resulting from an internal partial solution tension. The action capacities causing exudation are equally operative under conditions of rapid water loss from aerial organs by evaporation. The

latter situation is merely characterized by the rate of evaporation exceeding the rate of water supply through the osmometer membrane. The movement of water, primarily through the tension, *i.e.*, the extrinsic hydrostatic specific free energy decrement, set up by evaporation (caused by reduced aerial vapor pressure), is accompanied by increased imbibitional specific free energy and increased capillarity associated with the forces of cohesion between water molecules, and are here a part of the tension expressed as an extrinsic hydrostatic specific free energy decrement, $(-Fh_1'')$, resulting from an internal partial solution tension.

The Individual Cell, Plasmolysis, etc.: Considering a reference cell system alone, many of the factors previously discussed come into action in the movement of the water from the external medium to the vacuole. The internal hydrostatic pressure is here expressed by turgor. When the osmometer is an inclosed system, the internal evaporation factor is excluded. For the isolated cell system in its initial state, the relations possibly involved in water transfer to the vacuole may be expressed as

$$NIF = Fs_i - Fh_1' \quad (11)$$

$$20.0 = 20.0 - 0 \quad (11a)$$

or as

$$NIF = (Fs_i + Fm) - (Fh_1' + Fs_e) \quad (20)$$

$$20.0 = (20.0 + 4.0) - (0 + 4.0) \quad (20a)$$

On entry of water to completely satisfy differences of free energy of water, these equations would assume the relations

$$0 = 16.0 - 16.0 \quad (26, \text{ see } 11c)$$

and $0 = (16.0 + 4.0) - (16.0 + 4.0)$, respectively. (27)

To cause plasmolysis, the water content of the internal phase would have to be reduced, through application of an efflux action capacity, to such a value that the internal hydrostatic specific free energy would be diminished to zero. As a consequence the osmotic specific free energy related to the presence of internal solute (or the solute concentration of the internal solution) would be increased. For the isolated reference cells just discussed, osmotic specific free energies, related to the presence of external solute, of 20.0 and 24.0 atmospheres, respectively, would be required to attain incipient plasmolysis.

Thus $NIF = Fs_i - (Fh_1' + Fs_e) \quad (28)$

$$0 = 20.0 - (0 + 20.0) \quad (28a)$$

$$\text{and} \quad \text{NIF} = (F_{S_1} + F_m) - (F_{h_1}' + F_{S_0}) \quad (20)$$

$$0 = (20.0 + 4.0) - (0 + 24.0) \quad (29)$$

The Individual Cell: Relationships in a Tissue: If the reference cell is surrounded by other cells, as in a tissue, intercellular pressure or tension, represented by a modified internal hydrostatic pressure, may be involved in the net influx of water. Under positive intercellular pressure conditions, osmotic turgidity of the encircled reference cell is attained by a smaller entry of water than where the reference cell is isolated¹⁸. On the other hand, when intercellular tensions are operative, a progressively increased internal hydrostatic tension obtains, for a similar reduction in volume of the inner phase of the reference cell. A positive intercellular pressure, resulting in an extrinsic internal hydrostatic specific free energy (F_{h_1}'' a ΔEF), equal to four atmospheres, for example, might arise on influx of water into the cell aggregate or tissue. For the reference cell in its initial state, in equation 11a

$$\text{NIF} = F_{S_1} - F_{h_1}' \quad (11)$$

$$20.0 = 20.0 - 0 \quad (11a)$$

such a pressure would give the following relations at water equilibrium, namely,

$$\text{NIF} = F_{S_1} - (F_{h_1}' + F_{h_1}''), \text{ where } (F_{h_1}' + F_{h_1}'') = F_{h_1} \quad (30)$$

$$0 = 16.6 - (12.6 + 4.0) \quad (30a)$$

Under conditions leading to water depletion the intercellular pressure may exist as a negative pressure (intercellular tension) resulting in an extrinsic specific free energy decrement, tending to cause water to move into the reference cell. Likewise, the resultant internal hydrostatic specific free energy may assume "negative" values ($-F_{h_1}$) leading through incipient cell plasmolysis to permanent injury, if water requirements are not satisfied. Incipient plasmolysis of the reference cell, when surrounded by other cells, would be accomplished by an external solution of solute concentration equivalent to that which plasmolysed the isolated reference cell. Here, equation 28a

$$\text{NIF} = F_{S_1} - (F_{h_1} + F_{S_0}) \quad (28)$$

$$0 = 20.0 - (0 + 20.0) \quad (28a)$$

would read

$$\text{NIF} = F_{S_1} - ((F_{h_1}' + F_{h_1}'') - F_{S_0}) \quad (31)$$

$$0 = 20.0 - ((0 + 0) - 20.0),$$

¹⁸ Where the reference cell is not completely encircled, but impinged upon only in limited areas by other cells, merely minor deformations may occur. The volume relations then approach those of the isolated reference cell.

where $Fh_1' + Fh_1'' = Fh_1$;

or its equivalent,

$$NIF = (Fs_1 + ((-Fh_1') + (-Fh_1'')) - Fs_0 \quad (32)$$

$$0 = (20.0 + (0 + 0)) - 20.0, \quad (32a)$$

where $(-Fh_1') + (-Fh_1'') = (-Fh_1)$.

Plasmolysis, beyond the state of incipience of the reference cell, to a relative volume of 0.975 would require external solutions of concentrations related to osmotic specific free energies of 22.4 and 23.4 atmospheres, respectively, for the isolated and the encircled reference cells. The equations for this would read

$$NIF = (Fs_1 + (-Fh_1')) - Fs_0, \text{ for an isolated cell, } (33)$$

$$0 = (20.8 + 1.6) - 22.4;$$

and $NIF = (Fs_1 + ((-Fh_1') + (-Fh_1'')) - Fs_0, \text{ for an } (32)$
encircled cell, where $(-Fh_1') + (-Fh_1'') = (-Fh_1)$,

$$0 = (20.8 + (1.6 + 1.0)) - 23.4 \quad (32b)$$

It should be noted from these considerations of the reference cell involving intercellular pressure or tension that the bounding cells impose pressures, resulting in hydrostatic specific free energies which are not linear with changes in volume. As a consequence, positive or "negative" internal hydrostatic specific free energies may be represented ideally, depending upon the circumstances, by either a linear function or a sigmoid curvilinear function. In the plant as a whole both types of functions may be operative simultaneously in the two reference osmotic systems¹⁹.

¹⁹ The animal cell may be treated similarly. In this case, however, we do not deal with a reinforcing cell wall and the concomitant effects of adhesion between the wall and the cytoplasmic membrane. Therefore, similar equations and diagrams are employed for the animal cell whether isolated or within a tissue, except that the mathematical relations are restricted to the first quadrant of the graph; (the plant cell may involve relations within the first, second and third). The relative volume of one, then represents zero volume of the internal phase. The same features would apply to the plant cell if the protoplast and inclusions were completely free of interconnection with the encircling wall. The animal cell does not possess a prominent discernible central vacuole (see 68.) The true internal volume cannot be ascertained. It is necessary in computations, therefore, to employ the volume of the cell as a whole which will include a non-solvent volume (including the membrane itself) occupied by lipids, proteins and other material that set up little or no antiphasic osmotic "solute" specific free energy of themselves. In this case the volume at minimal hydrostatic specific free energy (obtainable only through extrapolation) will not be zero, as required for the internal phase of the osmometer, but will be a real quantity. This value will be a measure of that part of the total space of the normal system measured, represented by the non-solvent volume.

Effect of External Salinity on the Open Reference System: Equations 16, 17 and 18 showed that an exudation at an effective height approximating 180 feet, equivalent to 5.3 atmospheres internal hydrostatic specific free energy or pressure, could be stopped by the presence of an external solution of solute concentration related to an osmotic "solute" specific free energy of 13 atmospheres and a tendency toward water efflux where solutions of greater solute concentration are involved. As where water evaporation occurs from the shoot of a plant (see discussion of equation 24), so also here, where a tension is applied externally, the efflux of water from the inner phase of the osmometer is not accompanied by a significant reduction in the height of the water column, but more likely by a lateral reduction in diameter.^{19a} The net result of this outward flow of water is the maintenance of the intrinsic internal hydrostatic specific free energy at approximately 5.3 atmospheres and the establishment of an internal partial solution tension, *i.e.*, an extrinsic hydrostatic negative specific free energy component. An external solution of solute concentration related to an osmotic "solute" specific free energy of 20.0 atmospheres would modify equation 17,

$$\text{NIF} = \text{Fs}_i - (\text{Fh}_i + \text{Fs}_e) \quad (12)$$

$$0 = 18.3 - (5.3 + 13.0) \quad (17)$$

$$\text{to read} \quad -7 = 18.3 - (5.3 + 20.0) \quad (34a)$$

tending to cause water to move outward with consequent reduction in volume of the internal phase. At water equilibrium equation 34a would become

$$0 = 20.0 - (0 + 20.0) \quad (34b)$$

in which the internal hydrostatic specific free energy is equal to $5.3 - 5.3 = 0$, *i.e.*, $(\text{Fh}_i' + (-\text{Fh}_i'')) = \text{Fh}_i$. For the plant these conditions of water equilibrium and of zero hydrostatic specific free energy (equation 34b) would represent, in a water saturated aerial environment, the condition of incipient permanent wilting.

Soil Effects (compare 17, 18, 25, 33, 41, 58, 63, 65): The discus-

^{19a} In general, the decrease in height of fluid, from the maximum effective height of the plant or organ, in an individual more or less anastomosed hydraulic duct, will probably be limited to the initial cross wall or the vertical functional length of the first apical trachea or tracheid, but may extend under some circumstances beyond these lower transverse vascular walls. In some species of angiosperms the effective reduction in the weighted average height, of a reference unit cross section, of the hydrostatic system may be of large magnitude. The intrinsic hydrostatic specific free energy will be reduced accordingly.

sion thus far has been concerned with an external medium of water or solution of unlimited volume. Where the soil represents the external phase of the plant osmometer, specific free energies of water other than those arising from an external solute concentration of solution are generally involved. This will be particularly true at soil moisture percentages below field capacity (18). These additional non-metabolic specific free energies (F_{nm_e}) restrain the flow of water inward, or, if of sufficient magnitude, may cause the outward passage of water through the membrane. This efflux specific free energy may include action capacities associated with reduced vapor pressure, imbibition, adsorption, capillarity and the like.

If the integrated plant osmometer be considered as exposed inwardly to a water-saturated aerial environment and separated outwardly by a soil at the permanent wilting percentage or the wilting coefficient (where the solute concentration of the soil solution is assumed, for example, to be related to an osmotic "solute" specific free energy of four atmospheres), the osmotic relations for such a system at water equilibrium would be modified from equation 34b

$$NIF = F_{s_i} - (F_{h_i} + F_{s_e}) \quad (12)$$

$$0 = 20.0 - (0 + 20.0) \quad (34b)$$

to read $NIF = F_{s_i} - (F_{h_i} + F_{s_e} + F_{nm_e}) \quad (35)$

$$0 = 20.0 - (0 + 4.0 + 16), \quad (35a)$$

or if the plant were capable of exerting a metabolic influx specific free energy of, for example, four atmospheres, then

$$NIF = (F_{s_i} + F_m) - (F_{h_i} + F_{s_e} + F_{nm_e}) \quad (36)$$

$$0 = (20.0 + 4.0) - (0 + 4.0 + 20.0) \quad (36a)$$

The plant, in these two cases, would be at incipient permanent wilting. The total efflux specific free energy due to conditions in the external medium, the soil, would equal in equation 35a, $4 + 16 = 20.0$ atmospheres; equation 36a, $4.0 + 20 = 24.0$ atmospheres.

Under ordinary conditions the plant normally may be exposed at the same time to both soil osmotic factors (salinity, imbibition, water depletion, *etc.*) and aerial factors (rapid evaporation from the shoot). The plant will suffer under stressed conditions, from an unfavorable net influx specific free energy or, over an extended interval of time, from an unfavorable rate of water supply due to a reduced or inadequate membrane permeability or, directly or in-

directly due to unfavorable metabolism of the organism. Provided the permeability of the membrane remains favorable and an adequate supply of water is maintained, rapid evaporation of water from the shoot should not prove particularly harmful with regard to the plant's water relation (63, p. 614). (Note its effect on the net influx specific free energy, other conditions being favorable (29)²⁰.) The chief causes of stress are related to water deficit or non-metabolic efflux specific free energies originating in the soil, and membrane permeability. If an additional internal hydrostatic negative specific free energy, resulting from an internal hydrostatic solution tension equal to 1.6 atmospheres, for example, is produced due to excessive water loss from the internal phase of the osmometer through aerial evaporation, equations 35a and 36a would become, respectively:

$$\text{NIF} = (\text{Fs}_i + (-\text{Fh}_i)) - (\text{Fs}_e + \text{Fnm}_e) \quad (37)$$

$$2.4 = (20.8 + 1.6) - (4.0 + 16) \quad (37a)$$

$$\text{and} \quad \text{NIF} = (\text{Fs}_i + \text{Fm} + (-\text{Fh}_i)) - (\text{Fs}_e + \text{Fnm}_e) \quad (38)$$

$$2.4 = (20.8 + 4.0 + 1.6) - (4.0 + 20.0) \quad (38a)$$

Wilting and Death: With respect to the water relations of the plant, the coördinated functioning of its various organs is essential to optimum growth under any set of environmental conditions. Water movements are directly or indirectly dependent on the integrated state of the meristems, sorbing and eliminating, conducting, dermal, ventilating and mechanical systems. The presence of intercellular pressures may be of advantage in the maintenance of rigidity of an organ. Under conditions of water deficit, extrinsic hydrostatic negative specific free energies, resulting from intercellular tensions, may be a detriment. The physiological anatomy and internal solution concentrations of the plant are generally adapted to accord favorably with its habitat of growth. A favorable water balance is thus provided. The solute contents of cells are graded to maintain the normal functioning of the organism as an integrated whole, even under limited stress with respect to water supply and demand. Wilting, within the limits of osmotic turgidity and zero internal hydrostatic specific free energy, during limited intervals of time, may not in itself prove lethal, directly or indirectly, to the plant.

²⁰ If the diameters of the vessels are materially reduced (likewise modifying surface tension) under the internal partial solution tensions, however, the absolute rate of water flow will be significantly reduced.

Permanent wilting of a plant may occur if, over an indefinite period of time, the supply of water is insufficient to supply the needs of the organ for growth and maintenance of a positive internal hydrostatic specific free energy. It may be considered to be in the state of incipient permanent wilting if recovery from zero internal hydrostatic specific free energy is not accomplished within a reasonable interval of time, when the plant is placed ideally into a water-saturated atmosphere, assuming approximate equilibrium within the body as a whole. If the plant in this state is in a soil approaching water equilibrium within itself, the soil is considered at the permanent wilting percentage (45, pp. 257-262). Most plant species, as a general rule, will exhibit permanent wilting²¹ at approximately the same moisture percentage of a particular soil. This observation is probably related primarily to the rapid decrease in free energy of the water in a soil as the water content of the soil decreases on passing through the wilting percentage of the soil (17, 33, 34, 45, 65; compare 25). Any differences among separate plants or species with regard to their incipient permanent wilting under the same environmental situation will be related to differences in their inherent net influx specific free energies and their relative permeabilities. Permanent wilting and irreparable injury to cells or an organ has been related to rupture of the continuity of the water conduits. Others have related death from desiccation to destructive effects of mechanical disturbances on the structure of living protoplasm. Parallel injury effects may arise from solute toxicity (41, 65).

Significance of Transpiration: There is no *a priori* reason for those specific free energies, which may be applied toward producing exudation, being unable to move water to the height of the tallest plant, under appropriate conditions. However, most of the available experimental data do not favor this possibility (34). The specific free energy related to internal solute is not correlated with the height of the plant or species. The specific free energies, due to the presence of internal solute, of greater magnitudes are more frequently associated with xerophytic or halophytic species. Mano-

²¹ In some species, evident wilting (drooping or curling of leaves or organs) does not characterize severe water deficit. Change of color of the leaves or a marked decrease in the rate of growth of the plant may indicate the exhaustion of readily available water. Desiccation below incipient permanent wilting for brief periods may not result in any apparent changes in the plant. The time factor and the rate of depletion of the available moisture are important (63).

metric pressures, equivalent to hydrostatic water columns 86 feet high, have been observed from decapitated roots of black birch (*Betula lenta*) under otherwise normal conditions. Using root tips of tomato, grown atypically by the tissue culture technique, White has reported inward movement of water against manometric pressures equivalent to a hydrostatic water column 200 feet tall. The flow was such as to indicate a dependence on respiratory processes (67). The coördinated regulation of water flow into the plant, at adequate rates, probably involves all of the possible influences previously discussed, including evaporation, *i.e.*, transpiration (8, 28, 34, 45).

Comprehensive Osmotic System of the Plant: For simplicity of discussion, the two reference systems have been treated using values

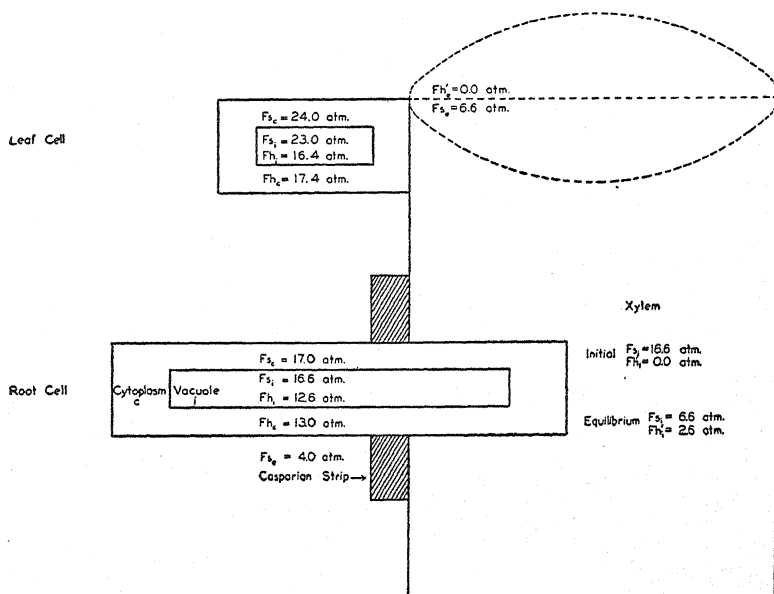


FIG. 4. The comprehensive osmotic system of the plant.

of like magnitudes for specific free energy and for volume changes. The water relations of the cytoplasm of a cell had not been analysed. The water relations of a typical leaf cell were not specifically evaluated. These concerns may be briefly discussed by values expressed in equations referring to Figure 4. The quantities related to the endodermal cell are in accord with those used earlier. The numbers recorded for the xylem, at water equilibrium, are smaller. This

assumes a larger change in volume for this internal phase, compared with that for a vacuole of a cell, on passing from minimal to maximal internal hydrostatic pressure.

A. Water relations of the endodermal cell of the root in a water-saturated environment:

a. At water equilibrium; cytoplasm (c); external medium (e)

$$\text{NIF} = (F_{s_e}) - (F_{h_c} + F_{s_e}) \quad (39)$$

$$0 = (17.0) - (13.0 + 4.0) \quad (39a)$$

b. At water equilibrium; cytoplasm (c); vacuole (i)

$$\text{NIF} = (F_{s_i} + F_{h_c}) - (F_{h_i} + F_{s_e}) \quad (40)$$

$$0 = (16.6 + 13.0) - (12.6 + 17.0) \quad (40a)$$

c. At water equilibrium; vacuole (i): external medium (e)

$$\text{NIF} = (F_{s_i}) - (F_{h_i} + F_{s_e}) \quad (41, \text{ see } 12)$$

$$0 = (16.6) - (12.6 + 4.0) \quad (41a, \text{ see } 12c)$$

It may be noted that this equation is equal to the sum of states represented by phases including the intermediate cytoplasm, *i.e.*, the sum of the two immediately preceding equations, as follows:

$$\text{NIF} = (F_{s_e}) - (F_{h_c} + F_{s_e}) \quad (39)$$

$$\text{NIF} = (F_{s_i} + F_{h_c}) - (F_{h_i} + F_{s_e}) \quad (40)$$

$$\text{NIF} = (F_{s_i}) - (F_{h_i} + F_{s_e}) \quad (41)$$

B. Water relations of the integrated system of the endoderm²²:

a. Initial state; external medium (e): internal xylem medium (i).

$$\text{NIF} = (F_{s_i}) - (F_{h_i}' + F_{s_e}) \quad (41)$$

$$12.6 = (16.6) - (0 + 4.0) \quad (41b)$$

b. At water equilibrium (other forces excluded); external medium (e): internal xylem medium (i).

$$\text{NIF} = (F_{s_i}) - (F_{h_i}' + F_{s_e}) \quad (41)$$

$$0 = (6.6) - (2.6 + 4.0) \quad (41c)$$

C. Water relations of a leaf cell in a water saturated aerial environment:

a. At water equilibrium; cytoplasm (c): external xylem medium (e).

²² The presence of an effective Casparian strip may not be considered by some to be indispensable to osmotic water movement in the integrated plant osmometer, but it certainly aids directly or indirectly in the establishment of an internal hydrostatic pressure of high order, at least under some circumstances (compare 34 and 52).

$$\text{NIF} = (\text{Fs}_e + \text{Fh}_e') - (\text{Fs}_e + \text{Fh}_e) \quad (42)$$

$$0 = (24.0 + 0) - (6.6 + 17.4) \quad (42a)$$

- b. At water equilibrium; cytoplasm (c): internal vacuole medium (i).

$$\text{NIF} = (\text{Fs}_i + \text{Fh}_e) - (\text{Fs}_e + \text{Fh}_i) \quad (43)$$

$$0 = (23.0 + 17.4) - (24.0 + 16.4) \quad (43a)$$

- c. At water equilibrium; vacuole: xylem. As in the preceding part A, these relations are equal to the sum of the conditions represented by phases including the intermediate protoplasm.

$$\text{NIF} = (\text{Fs}_i + \text{Fh}_e') - (\text{Fs}_e + \text{Fh}_i) \quad (44)$$

$$0 = (23.0 + 0) - (6.6 + 16.4) \quad (44a)$$

Where the aerial environment is not saturated with respect to water, a non-metabolic action capacity may arise due to evaporation, tending to cause water to move out of the leaf cell. Water relations of the xylem will be concurrently modified. Deviations from a water-saturated external medium bathing the root have been discussed hereinbefore.

In the above discussion of the comprehensive osmotic system of the plant, possible free energies related to metabolism have not been considered. The hypothetical specific free energy values used are related to the weighted average solute concentrations of the phase in question. The specific free energies related to the solute concentrations of the vacuolar solutions may not be in full accord with the viewpoints of certain investigators. Some believe that the vacuolar fluids of leaf cells may be very dilute (42, compare 16 and 68). They lay major responsibility for the influx of water on a possible metabolic specific free energy. The figures employed herein merely exemplify one opinion.

In certain commercial and experimental procedures plant fluids are obtained from wounded surfaces of aerial portions of plants as the result of scathing. The release of exudate or bleeding fluid by this means may result from an increased solute concentration at the externally effective surface. Since one or more differentially permeable membranes are probably operative between the injured surface and the internal phase of the reference osmometer, a decreasing specific free energy for water may be maintained across the intermediate surfaces by the repeated injury, through con-

tinuous disturbance of the osmotic equilibria. Generally, a concomitant hydrostatic pressure within the tracheal system has been reportedly absent. In any system water will tend to flow toward the phase or region of the lowest specific free energy. In the absence of positive hydrostatic pressures, other conditions being equal, this exudation, considered to be an efflux, would tend to occur if the lowering of the specific free energy of water due to the treatment (F_{s_0} or ΣEF) is greater in magnitude than the concurrent total influx specific free energy (ΣIF), at the same level in space.

So-called "glandular secretion" may be a natural manifestation of a similar phenomenon from uninjured tissue. In this case, a metabolic polarized movement of solute must be postulated, toward the cytoplasmic surface where the resultant exudate appears, followed or accompanied by an osmotic flow of water. A unidirectional metabolically governed flow of water has not been proven, but it is not excluded as a possibility.

The phenomenon of sap flow from red maple in early spring has been reinvestigated (55). The ultimate flow of sap is probably a mass flow of solution along lines of least resistance on removal of restraint when the hydraulic system is tapped. At times when those osmotic specific free energies which generally combine to cause exudation are ineffective (transpiration nil), temperature differences within the plant, which always exist but are considered constant elsewhere in this thesis, may play a more prominent or dominant rôle in the movement of water. Stevens and Eggert correctly suggested that as partial freezing of water occurs, water will tend to move toward this region due to a lowering of the free energy of water. This lowering is caused by two factors, lowering of the temperature *per se* and an increase of solute concentration in the remaining solution. Assuming an available source of water, *e.g.*, in the lower root regions of the soil, water will flow toward the location of partial freezing. This process will be accompanied, to some extent at least, by partial dehydration of the individual cells. The free energy difference for water is related to the degree of freezing and its accompanying effects. Ice crystallization probably proceeds in the following order: water in intercellular spaces, cell vacuoles and tracheal ducts, and finally protoplasm. In normal winter freezing this sequence is not completely accomplished, since

favorable cytoplasmic relations must be maintained for renewed growth as the season advances favorably. The release of appreciable amounts of solute (especially sugar) with the water as a flow of sap probably implies some cell destruction or at least an altered permeability and/or metabolism of cells. On thawing, an excess hydrostatic pressure will tend to cause water or solution (water and solute) to move through open channels affording a flow of sap.

Condensation of Water at Surfaces: The appearance of liquid at a surface may be due to two distinct phenomena which should be discriminated one from the other (compare 1). The first may be related to exudation from an organism as exudate, especially, for example, guttation fluid. This is caused by the existence of a net influx specific free energy as an over-all process by the integrated osmotic system of the plant. The second phenomenon may be related to condensation of water vapor at an exposed surface of the organism, as for example, dew. This may be caused by the lowering of the free energy of the water molecules attending energy transfer from these particles to the surface, when the surface is at or below the moisture saturation temperature. Such a condensation may be increased by the presence of solute at the interface through an enhanced lowering of the free energy of the water molecules. This amassing of fluid will occur under conditions otherwise favorable for guttation. It may also arise if the rate of amassment is greater than that of a possible rate of water flux into the plant. Continued heaping of fluid in the latter way may account, in part, for the dripping of solution from aerial organs of tree species such as *Lonchocarpus florus* and *Liveetia elegans* under conditions otherwise unfavorable for guttation. Water depositions of this kind occur in the tropical rain forest area of Latin (Hispanic) America. The overflow of collected water may or may not be assisted by capillary activity of external integuments of leaves.

Effect of Temperature (see 34, 44) : In this treatise the temperature relations of the system have been considered to be constant. Changes in temperature under real situations will admittedly affect several of the factors involved in the flow of water. Rise in temperature will increase primarily

- (a) the free energies of the solute and solvent molecules of solutions;

- (b) the rate of evaporation (through lowered aerial vapor pressure), hence the internal partial or total solution tension, through lowering of the vapor pressure of water in the extra-phasic environment;
- (c) the rate of metabolism which may enhance, first, the rate of formation of osmotically active metabolites, and second, the release of energy for possible direct or indirect metabolic specific free energies;
- (d) the product of pressure and volume within a phase.

Temperature changes may influence the rate of water movement by modifying the viscosity of the fluid, which is a minimum at 4° C.

Miscellaneous Effects: Under experimental conditions still additional influences related to water flow (see also p. 27, suction effect) may be artificially applied to the osmotic system either externally or internally or both, *e.g.*, an applied electrical potential difference, or a differential mechanical or gas pressure. The constituent specific free energy (ΔIF or ΔEF) would tend to move water unilaterally, similarly to that by a possible metabolic specific free energy. In the latter case, however, the osmotic influence is not artificially modified, *e.g.*, by narcosis, *etc.*

Concluding Statements: Heretofore in this treatise the cytoplasm of the reference cell or of the protoplasm of the integrated system has been assumed to be homogeneous in its properties and of uniform metabolism. As such, it was viewed, *in toto*, as a differentially permeable osmometer membrane²³.

These relationships no doubt differ in space within the ectoplasm, mesoplasm and endoplasm, and with time. Differences in the specific free energy of solute and solvent particles within these regions would be expected to exist. If a decreasing solute concentration is maintained across the protoplasm from the ectoplasm to the endoplasm of either system, water equilibrium could be readily attained through a lowered hydrostatic specific free energy within the system as a whole. Since, in any case, the difference of osmotic

²³ Especially in cells in which a central vacuole is not apparent, as for example in meristems, it is possible that a dispersed system of minute osmometers may exist within the cytoplasm. The theories of Duclaux; Proctor and Wilson, and Loeb (30, p. 122; 9, p. 333) may have practical application here. It is suggested that gel walls act as membranes which surround dissolved molecules or extremely small colloidal particles. It is assumed that limited swelling occurs accompanied by elastic stretching of the gel walls in opposition to an internal hydrostatic pressure, in response to an influx of fluid caused by an established net influx specific free energy.

"solute" specific free energy through the membrane itself would be small, the net specific free energy tending to cause water to move into the integrated system would be very closely expressed by the relative osmotic "solute" specific free energies and hydrostatic specific free energies related to the external and internal phases, with the protoplasm considered as a simplified homogeneous membrane.

Operation of the integrated endodermal system as discussed herein assumed prior equilibrium within the endodermal cell. Actually, satisfaction of water deficits in the two reference systems under discussion and within a cell series may proceed simultaneously (compare 34 with 6 and 59).

In the plant as a whole, water will tend to move with the direction in which its specific free energy decreases. In any case, the tendency for water movement at any instant of time will be accurately expressed in terms of the net energy tending to cause water to move through the membrane of the osmometer, namely, the net influx specific free energy. The over-all rate of water movement (Part II) will depend on the product of the permeability of the membrane, and the net influx specific free energy gradient, in accordance with Poiseuille's or Torricelli's theorem (included in the permeability coefficient), as applied to the flow of liquids through tubes; the latter, depending upon which formula, if either, is more or less applicable (compare data of Lundegårdh, 39, and 7, p. 142).

EXPERIMENTAL EVALUATION OF OSMOTIC QUANTITIES

Summary of Osmotic Relations: A brief recapitulation of equations and reference states may be made as an introduction to an analysis of experimental methods for the estimation of osmotic values in certain type cases.

The osmotic specific free energy balance, tending to cause water to move into plants is expressed by the equation,

Net influx specific free energy = $\sum \text{Influx specific free energies} - \sum \text{Efflux specific free energies}$.

$$\text{NIF} = \sum \text{IF} - \sum \text{EF}$$

$$\text{NIF} = (\text{IF} + \Delta \text{IF}) - (\text{EF} + \Delta \text{EF}), \text{ or, for example,}$$

$$\text{NIF} = (\text{Fs}_1 + \text{Fm}) - (\text{Fh}_1 + \text{Fs}_e), \quad (20)$$

where $\text{Fh}_1 = \text{Fh}_1' + \text{Fh}_1''$, Fm and Fs_e are used as possible examples of additional influx and efflux specific free energies.

NIF is the net influx specific free energy tending to cause unbound (or solvent) water to move into the internal phase of the plant osmometer.

ΣIF is the algebraic sum of those osmotic specific free energies tending to cause unbound (or solvent) water to move across the semipermeable membrane into the internal phase of the plant osmometer; opposed to ΣEF .

ΣEF is the algebraic sum of those osmotic specific free energies tending to cause unbound (or solvent) water to move across the semipermeable membrane into the external phase of the osmometer.

F_{s_i} is the antiphasic osmotic specific free energy related to the solute concentration internally; see C_i .

F_{s_e} is the antiphasic osmotic specific free energy related to the solute concentration, externally; see C_e .

C_i is the internal solute concentration related to F_{s_i} .

C_e is the external solute concentration related to F_{s_e} .

F_m is a possible metabolic specific free energy, here inwardly directed.

F_{h_i} is the internal hydrostatic specific free energy equal to the algebraic result between the intrinsic and extrinsic hydrostatic specific free energies.

Since in any biological osmotic system F_{s_i} and F_{h_i} are fundamentally involved, they have been designated as IF and EF, respectively, in the general equation. F_m and F_{s_e} may or may not apply in any specific case, and are therefore symbolized in a general way by ΔIF and ΔEF .

V is the volume of the internal phase of the osmometer in arbitrary units (the vacuolar, or xylem and possibly intercellular space within the endodermis, is essentially implied for the two systems discussed).

e and i are subscripts referring to the external and internal phases, respectively, of the reference osmometer; the regions within which the constituent influence, modifying the free energy of the component water molecules, may be imposed.

o , n and m are subscripts referring to the conditions of the osmometer at zero, intermediate normal or control, and maximal internal hydrostatic specific free energy (*i.e.*, that attainable when the external medium is water).

Δ is a symbol for a finite change in any osmotic value.

At zero internal hydrostatic specific free energy (*e.g.*, a cell at incipient plasmolysis) :

$$NIF_o = (Fs_{io} + Fm_o) - (Fs_{eo}) \quad (45)$$

since Fh_i is zero, here. At water equilibrium $NIF_o = 0$, therefore

$$Fs_{eo} = (Fs_{io} + Fm_o) \quad (46)$$

At normal, or control internal hydrostatic specific free energies :

$$NIF_n = (Fs_{in} + Fm_n) - (Fh_{in} + Fs_{en}) \quad (47)$$

At water equilibrium $NIF_n = 0$, therefore

$$Fs_{en} = (Fs_{in} + Fm_n) - Fh_{in} \quad (48)$$

or

$$Fh_{in} = (Fs_{in} + Fm_n) - Fs_{en} \quad (49)$$

At maximal internal hydrostatic specific free energy (external phase pure liquid, water) :

$$NIF_m = (Fs_{im} + Fm_m) - (Fh_{im}) \quad (50)$$

since Fs_{em} is zero, here. At water equilibrium $NIF_m = 0$, therefore

$$Fh_{im} = (Fs_{im} + Fm_m) \quad (51)$$

Procedures for the Estimation of Osmotic Quantities (see 57):

There are two general means of approach to these values; the first, dealing with water equilibria, involves the following techniques :

1. Measurement of the internal volume of the osmometer at zero, normal and maximal internal hydrostatic specific free energy (resulting from the corresponding pressure) of the system.
2. Determination of the solute concentration of the external medium of the osmometer at water equilibrium, for the system at zero and normal internal hydrostatic specific free energies; and/or the related (antiphasically effective) osmotic specific free energy, by calculation (see 10), or by cryoscopic (14, 22, 24) or vapor pressure (21) methods.
3. Determination of the osmotic "solute" specific free energy related to the presence of solute in the internal medium²⁴ of the osmometer, by cryoscopic, or vapor pressure, or hydrostatic pressure (22, 48) methods.

²⁴ By determining the specific conductance (volume conductivity, in reciprocal Ohm-centimeters, *i.e.*, mhos per centimeter, L) of the fluid, the ratio of the specific conductance to the comparable freezing point depression (in degrees absolute lowering, ΔT_f), can be obtained. This ratio ($L/\Delta T_f$) is an expression for the relative abundance of electrolytes as compared with the total solutes (electrolytes and non-electrolytes) present in the medium (23).

4. Estimations of certain other osmotic quantities by a special technique (2); see case B, following.

By these means, values may be obtained for most of the quantities desirable for an inspection of the system as a whole, whether a reference cell or the integrated (open inner phase) plant osmometer.

The second approach is of value only for the integrated plant osmometer system. Here, non-equilibria for water are primarily concerned. The following techniques are involved:

1. Measurement of the relative rates of flow of liquid across the semipermeable membrane, generally made at or approaching zero internal hydrostatic specific free energy as modified by various solute concentrations in solutions applied to the external phase.
2. Determination of the solute concentrations of the external medium, and/or the related osmotic specific free energies (by methods as in item 2, above), at various rates of liquid flow as measured in item 1, immediately preceding.
3. Determination of the osmotic "solute" specific free energy related to the presence of solute in the internal medium of the osmometer as in item 3, above.

Any series of operations involves assumptions of various validity. The correctness of any assumption will depend upon a number of factors, especially on the system involved and the experimental approach. Some of the more important assumptions may be listed for the two major systems. The validity of each assumption may be reasoned from a detailed examination of the particular experimental situations involved in any set of data. Following are assumptions associated with the reference cell as the osmotic system:

1. Time is infinitesimal.
2. Temperature and metabolism (compare 5) are constants.
3. There is no movement of solute across the semipermeable membrane. The permeability of the membrane is constant to either solute or solvent with time.
4. The elastic modulus of the membrane is constant in space and time (compare 1).
5. The measured volumes of the internal phase of the osmometer are that of the reference cell vacuole at water equilibria. Where tissues are employed the cells are uniform, acting as individual entities, *i.e.*, intercellular pressures or tensions are absent. (Note,

measurements of tissue lengths involve errors embodied in assumptions 4 and 5.)

6. Incipient plasmolysis is accurately estimated. Adhesion between cytoplasm and wall is nil.

7. The determined solute concentration of the external medium is its weighted average concentration, bathing the membrane externally, at equilibrium (see footnote 10). The osmotic specific free energy related to the presence of external solute is accurately determinable²⁵. The concentration of the external medium is constant with time.

8. The osmotic specific free energy related to the presence of solute in the internal medium is accurately determined through the analytical technique and mathematical procedure employed (see footnote 25). (Note, composite expressed sap from whole cells involves obvious aberration, involving vacuolar, cytoplasmic and extra-cellular fluid associated with non-solvent (bound) water and modified solute effects.) Modification of the solute concentration with proportional change in volume is calculated from the ratios of the volumes to that of concentrations, not to that of osmotic "solute" specific free energies.

9. The osmotic specific free energies related to the presence of external and internal solute, are equal at osmotic water equilibrium, but only when the internal hydrostatic specific free energy or pressure is zero and if metabolic specific free energy is nil.

10. Hydrostatic specific free energy changes, internally, are proportional to the corresponding changes in volume of the internal phase of the osmometer. The hydrostatic specific free energy internally is zero at incipient plasmolysis.

11. The hydrostatic specific free energy, externally, is nil.

Following are assumptions associated with the integrated reference osmotic system:

1. Time is infinitesimal.
2. Temperature and metabolism are constants.
3. The permeability of the osmotic membrane is constant to either solute and solvent with time.

²⁵ This implies that the anti-phasically effective osmotic "solute" specific free energy mathematically obtainable from the related solute concentration in solution is accurately expressed by means of van't Hoff's equation (see equations 3, 3a and 3b), and that any essential, specific free energy, correction coefficient (see footnote 6) is accurately known and applied for the conditions experimentally imposed.

4. The rates of fluid flow through the semipermeable membrane into the inner phase of the osmometer, as modified by various solute concentrations applied to the external phase, are constant with time for each set of conditions.

5. The concentration of each of the solutions applied externally is constant with time. The determined solute concentration of the external medium is its weighted average concentration bathing the membrane externally (see footnote 10). The osmotic specific free energy related to the presence of external solute is accurately determinable (see footnote 25).

6. The osmotic specific free energy related to the presence of solute in the internal medium is accurately determined through the analytical technique and mathematical procedure employed (see footnote 25). This quantity is the weighted average value for the solution bathing the membrane internally (see footnote 10), corresponding to the determined rate of flow of fluid.

7. The hydrostatic pressures, internally and externally, are nil.

Obviously biological systems differ from these assumptions in varying degree. An observed or calculated osmotic quantity is the algebraic result of the possible deviations from the true value. The importance of these possible deviations can be realized, for example, from the controversy over the reality or non-existence of a metabolic specific free energy (active, vital, non-osmotic pressure) as a possible action capacity normally tending to cause water to move into plants.

Osmotic Quantities of a Cell: Two Type Cases: Case A: For those unfamiliar with the procedure followed in arriving at the values recorded in the literature for osmotic phenomena, a type case may be presented based on a recent study of Lyon (40), following essentially the plan of Ursprung (61). (Compare 4, 13, 42, 56, 66.)

1. The tissue volumes are obtained, from optical measurements, at zero (incipient or limiting plasmolysis of 50% of the cells), normal and maximal internal hydrostatic specific free energies (or corresponding pressures) of the cell. Thus

$$\begin{array}{rcccccccc} V_o & = & 2106 & \text{in arbitrary units, or } V_o & = & 1.000 & \text{in relative units} \\ V_n & = & 2380 & \text{"} & \text{"} & \text{"} & V_n & = & 1.130 & \text{"} & \text{"} & \text{"} \\ V_m & = & 2860 & \text{"} & \text{"} & \text{"} & V_m & = & 1.356 & \text{"} & \text{"} & \text{"} \end{array}$$

2. The sucrose solution (plasmolyte) concentration necessary to just plasmolyse 50% of the cells in the tissue is observed. Thus $C_{eo} = 0.70$ Molar (mols per liter of solution).

3. The antiphasic osmotic "solute" specific free energy related to the plasmolysing solution is estimated from item 2 by calculation (see 10; and item 7, page 48) or by means of conversion data (see 47, 48, 62). Thus $C_{eo} = 0.70$ M sucrose, is related to $F_{seo} = 21.5$ atmospheres. (Here, as in the calculations of Lyon (40), the conversion data of Molz (47) were used.) Where cryoscopic analyses are performed, the osmotic "solute" specific free energies are determined directly (24).

4. By observing the sucrose solution concentration as a bathing medium necessary to maintain the normal volume, or weight (see 46, 57), relationships within the tissue, C_{en} and F_{sen} , can be similarly estimated (see items 2 and 3). Lyon estimated $C_{en} = 0.32$ M and the related $F_{sen} = 8.7$ atmospheres.

5. At equilibrium between the vacuoles of the cell tissue and the plasmolysing solution the tissue could be treated by freezing and sap expression and the osmotic "solute" specific free energy, related to the solute concentration of the composite sap, determined by cryoscopic, vapor pressure or *in vitro* osmotic technique, as a measure of the antiphasic osmotic "solute" specific free energy related to the presence of internal solute. This estimate may be open to serious error due to admixture of fluids and modified by bound water effects (13). Thus C_{io} and F_{sio} could be found. Using tissue at normal internal hydrostatic specific free energy and at maximal internal hydrostatic specific free energy, C_{in} and C_{im} , and F_{sin} and F_{sim} can be similarly obtained.

On the *a priori* assumption that an inwardly directed metabolic specific free energy does not exist (which is not justified, but may yield an approximate measure of such a pressure from subsequent calculations) Lyon set $F_{seo} = F_{sio}$ and $F_{sen} = F_{sin}$ (see items 3, 4 and 5). Assuming that the internal solute concentration is inversely proportional to the change in volume of the inner phase, C_{in} and C_{im} can be calculated. Thus

$$\frac{C_{in}}{C_{io}} = \frac{V_o}{V_n} \text{ or } C_{in} = C_{io} \times \frac{V_o}{V_n} = 0.70 \times \frac{2106}{2380} = 0.620 \text{ Molar,} \quad (52)$$

and

$$C_{im} = C_{io} \times \frac{V_o}{V_m} = 0.70 \times \frac{2106}{2860} = 0.515 \text{ Molar.} \quad (52a)$$

By conversion, $F_{sin} = 18.5$ atmospheres, and $F_{sim} = 14.8$ atmospheres. The value F_{sin} can not be calculated directly from F_{sio} .

by using the quotient of the volumes as a factor (compare 40 and 61), because the related osmotic "solute" specific free energy changes at a greater rate, represented by a hyperbolic function, than does the concentration (61; 57, pp. 51-56).

6. The internal hydrostatic specific free energy at maximal turgor is computed from the equal and opposing action capacity, the osmotic specific free energy related to the presence of internal solute, at maximal turgor. Thus, $F_{h_{im}} = F_{s_{im}} = 14.8$ atmospheres. The internal hydrostatic specific free energy at minimal turgor or turgidity is zero by definition. Thus, $F_{h_{io}} = 0$ atmospheres. In the absence of intercellular (tissue) pressure or tension, the internal hydrostatic specific free energies at normal turgor can be computed from the proportional change of internal hydrostatic specific free energy with internal volume. Thus

$$\frac{F_{h_{in}} - F_{h_{io}}}{F_{h_{im}} - F_{h_{io}}} = \frac{V_n - V_o}{V_m - V_o}, \text{ but since } F_{h_{io}} \text{ is zero, then}$$

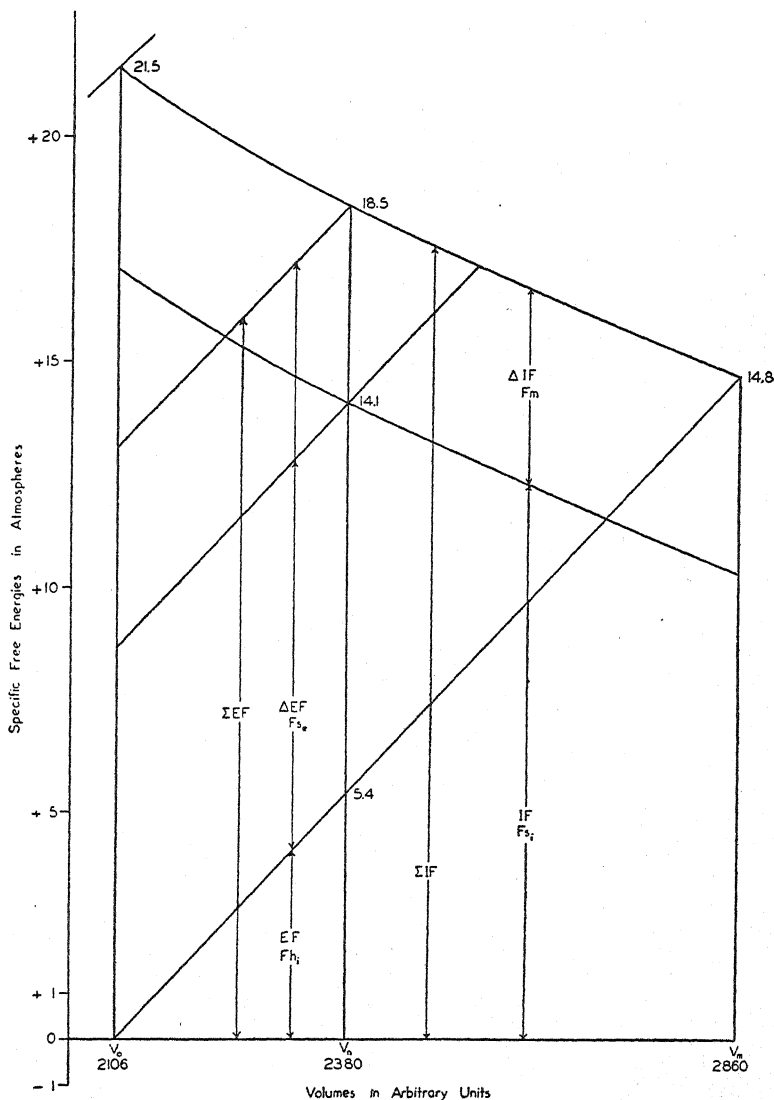
$$F_{h_{in}} = F_{h_{im}} \times \frac{V_n - V_o}{V_m - V_o} = 14.8 \times \frac{2380 - 2106}{2860 - 2106} = 5.4 \text{ atmospheres. (53)}$$

7. It was assumed (under item 5, which see) that a possible inwardly directed metabolic specific free energy was non-existent. $F_{s_{en}}$, under item 4, was estimated to be 8.7 atmospheres. (In Figure 4, $F_{h_{in}} + F_{s_{en}} = \Sigma EF$ or $5.4 + 8.7 = 14.1$). However, from the following calculation (data from items 5 and 6)

$$F_{s_{en}} = F_{s_{in}} - F_{h_{in}} = 18.5 - 5.4 = 13.1 \text{ atmospheres.}$$

The difference of 4.4 atmospheres between these values (8.7 atm. and 13.1 atm.) leads to the conclusion that an additional osmotic action capacity is operative in the system. This action capacity may obviously be realized as the metabolic specific free energy assumed to be non-existent in the earlier analysis of these data (see under item 5). The magnitude is an approximation from the mathematical process involved. These data, on the basis of the present scheme, are shown graphically in Figure 5. A more direct approach to this quantity would have been from determined values of the osmotic "solute" specific free energies related to the solute concentrations in the fluids from the external and internal phases of the osmometer system at minimal turgor and water equilibrium.

Then $NIF_o = (F_{s_{io}} + F_{m_o}) - (F_{h_{io}} + F_{s_{eo}})$ where NIF_o and $F_{h_{io}}$ are zero, $F_{s_{eo}} = (F_{s_{io}} + F_{m_o})$ or



OSMOTIC RELATIONS OF A POTATO TUBER CELL

$$[NIF = \Sigma IF - \Sigma EF = (F_{s1} + F_m) - (F_{h1} + F_{s2})]$$

FIG. 5. Water relations of a plant cell. The fundamental data were obtained, with slight modification (see text), from Lyon (40). The hyperbolic curves representing IF (*i.e.*, F_{s1}) and ΔIF (*i.e.*, F_m) are drawn parallel. This assumes that the metabolic specific free energy is constant under the experimental conditions.

$Fm_o = Fs_{eo} - Fs_{io}$; in other words, a positive difference between the determined values of the osmotic "solute" specific free energies, as written, would suggest in reality a possible inwardly directed metabolic specific free energy tending to cause water to move into the biological osmotic system.

Case B: The ingenious apparatus designed by Arens (2) would appear to afford exceptional opportunities for the measurement of certain osmotic quantities. By this technique a band of a single *Nitella* cell can be fixed in an impermeable metal collar. The arrangement may be likened to an individual endodermal cell encircled by its Casparian strip. The tractable terminal sections are enclosed by spaces which may be occupied by gases or liquids. In the latter situations this osmometer may be experimentally observed under such differential osmotic relations as may be applied to the opposite poles of the cell. The cell as a whole thus acts like a living semipermeable membrane, similar to the integrated osmotic system discussed for the entire plant. The system may also be viewed as a single cell; therefore, the arrangement affords systems of an isolated cell and an integrated osmometer at one and the same time.

The designer has subjected his experimental cell to an artificial situation where at least one of the spaces surrounding the free ends of the organ is filled with gas under pressure. The turgidity of the cell is observed by the angle of declination from the horizontal presented by the unrestricted portions of the filament. The flow analysis now proposed allows the experimental procedures of this case to be critically evaluated. At water equilibrium, the cell may, for example, assume the values (see Figure 3)

$$\begin{aligned} NIF &= \sum IF - \sum EF \\ NIF &= (Fs_i) - (Fh_i) \\ 0 &= (16.0) - (16.0). \end{aligned} \quad (54, \text{ see } 11c)$$

On evaporation of water from this cell, when subjected to a gaseous current of low moisture content in the external environment, the relative vacuolar volume might decrease from 1.5 to 1.085. This loss of turgor may then be represented by changes in the osmotic quantities to the values

$$13.0 = (18.3) - (5.3) \quad (55)$$

A differential of gas pressure is then rapidly applied (compare section entitled "Miscellaneous Effects") to the free portions of the

cell to restore the angle of declination, due to wilting, to zero. Accompanied by no increase in the volume of the cell vacuole, the net influx specific free energy would then approach zero, *i.e.*,

$$\begin{aligned} \text{NIF} &= (F_{s_i}) - (F_{h_i}) \\ 0 &= (18.3) - (18.3) \end{aligned} \quad (56)$$

F_{h_i} is equal to the sum of two specific free energies, an intrinsic and an extrinsic internal hydrostatic specific free energy, where

$$F_{h_i} = F_{h_i'} + F_{h_i''} = 5.3 + 13.0 = 18.3 \text{ atmospheres.}$$

The extrinsic internal hydrostatic specific free energy ($F_{h_i''}$ or ΔEF) arises in opposition to the externally applied pressure. In other words, the extrinsic internal hydrostatic specific free energy, here equal to 13.0 atmospheres, is the result of the applied gas pressure measured by the manometer or gas gauge.

Experimentally observed manometric pressures of 3–10 atmospheres have been reported (2). In the absence of a possible metabolic specific free energy, this osmotic quantity is also a measure of the net influx specific free energy for water when the cell was wilted. Contrary to report (2), this pressure is not a measure of the initial internal hydrostatic specific free energy of the cell at maximal turgor. This was actually 16.0 atmospheres (see equation 54).

Osmotic Quantities of an Integrated System: Two Type Cases:

Case A: The following data were selected from van Overbeek (40) in which the osmotic specific free energy balance was observed for decapitated plants at water equilibrium (compare 16 and 39).

1. The osmotic specific free energy related to external solute was calculated from cryoscopic measurements of the external medium which just prevented water movement through the membrane of the plant. The $F_{s_{eo}}$ thus found was 1.40 atmospheres.
2. The osmotic specific free energy related to the presence of internal solute was calculated from cryoscopic measurements of the exudate when the roots were bathed, over short intervals of time, with water. The $F_{s_{io}}$ thus found was 0.40 atmosphere.
3. Since the system was observed essentially at zero internal hydrostatic specific free energy, and at water equilibrium, a difference between these two quantities, $F_{s_{eo}}$ and $F_{s_{io}}$, may be

ascribed to an inwardly directed metabolic specific free energy. By difference this metabolic specific free energy is found to equal 1.00 atmosphere. (*i.e.*, $Fm_o = Fs_{eo} - Fs_{io} = 1.4 - 0.4 = 1.0$ atm.) In a similar type of experiment by Eaton (16) a metabolic specific free energy could not be realized, *i.e.*, the difference between Fs_{eo} and Fs_{io} was not significant. The osmotic analysis at water equilibrium was preferred over methods involving the rate of bleeding, in that changes in the water permeability of the root cells are not considered to affect these specific free energy determinations (compare case B, below).

Case B: The following data were selected from Sabinin (54) and Litvinov (36) in which the osmotic quantities were derived from the relative rates of bleeding from decapitated plants subjected to external media of known solute concentration (compare 39).

In the following equations similar symbols are employed as before. In addition, initial velocities of exudation are expressed by v_m , v_n and v_o representing, respectively, the velocities when the system is exposed to external phases of water, hypoosmotic and isoosmotic media. The character k is a permeability factor. This coefficient of proportionality (k) is a constant if the rate of exudation is not changed by a subsequent transfer of the root system into water following an experimental transfer from water to a solution. During brief intervals of time (less than 30 minutes), exudation velocities did not vary significantly (54).

Litvinov (36) made use of the following types of equations, assuming constancy of k , and Fs_1 and Fm , and obtained the corresponding figures, in one experiment:

$$v_m = (k_m) \cdot (NIF) \quad (57)$$

$$v_{n1} = (k_{n1}) \cdot (NIF) \quad (57a)$$

$$\text{and} \quad v_{n2} = (k_{n2}) \cdot (NIF) \quad (57b)$$

then

$$NIF = \frac{v_m(Fs_{en1})}{v_m - v_{n1}} = \frac{(14) \times (6.4)}{(14) - (3.5)} = 7.8 \text{ atmospheres} \quad (58)$$

$$\text{or} \quad NIF = \frac{v_m(Fs_{en2})}{v_m - v_{n2}} = \frac{(14) \times (7.6)}{(14) - (0.5)} = 7.9 \text{ atmospheres.} \quad (58a)$$

When the velocities are compared, where the root system is alternately bathed by two solutions of dissimilar concentrations (rather

than in the one case by water), use is made of the following relation, where

$$NIF = \frac{(v_{n1}) \times (Fs_{en2}) - (v_{n2}) \times (Fs_{en1})}{v_{n1} - v_{n2}} \quad (59)$$

$$NIF = \frac{(3.5) \times (7.6) - (0.5) \times (6.4)}{(3.5) - (0.5)} = 7.8 \text{ atmospheres.} \quad (59a)$$

The osmotic "solute" specific free energy, related to the solute concentration of the exudate when the roots were bathed in water, was obtained cryoscopically. This was compared with the net influx specific free energy determined independently. Placing figures expressed in atmospheres, cited from Table 3, p. 51 (36), into the appropriate equation:

$$NIF = (Fs_i + Fm) - (Fh_i + Fs_e) \quad (60)$$

$$\text{Where } Fh_i \text{ and } Fs_e \text{ are zero, then } Fm = NIF - Fs_i. \quad (61)$$

The following relations are obtained:

$$-0.25 = 0.43 - 0.67$$

$$-0.01 = 0.35 - 0.36$$

$$-0.04 = 0.38 - 0.42$$

$$+0.07 = 1.53 - 1.46$$

The results would suggest the absence of any significant metabolic specific free energy in the movement of water in these plants. It should be noted that negative evidence of this sort does not constitute proof of the non-existence of metabolic specific free energies in biological systems as a whole. Small positive figures, although suggestive of the presence of such action capacities, may be related to errors in technique.

SUMMARY

The fundamental principles of osmosis are reviewed. A scheme is presented for mathematically dealing with the osmotic quantities, expressed as osmotic specific free energies, and graphically representing the same in a diagram with appropriate coordinates. Theoretical cases are introduced, utilizing various osmotic action capacities which possibly control water movement between the plant and its environment. Four typical sets of data are analysed by the present scheme and limitations discussed.

A survey is made of methods currently employed in obtaining various osmotic quantities in plants.

The controversy as to the reality or non-existence of metabolic

specific free energies (variously termed active, vital or non-osmotic pressures) must await more critical experimentation.

ACKNOWLEDGMENTS

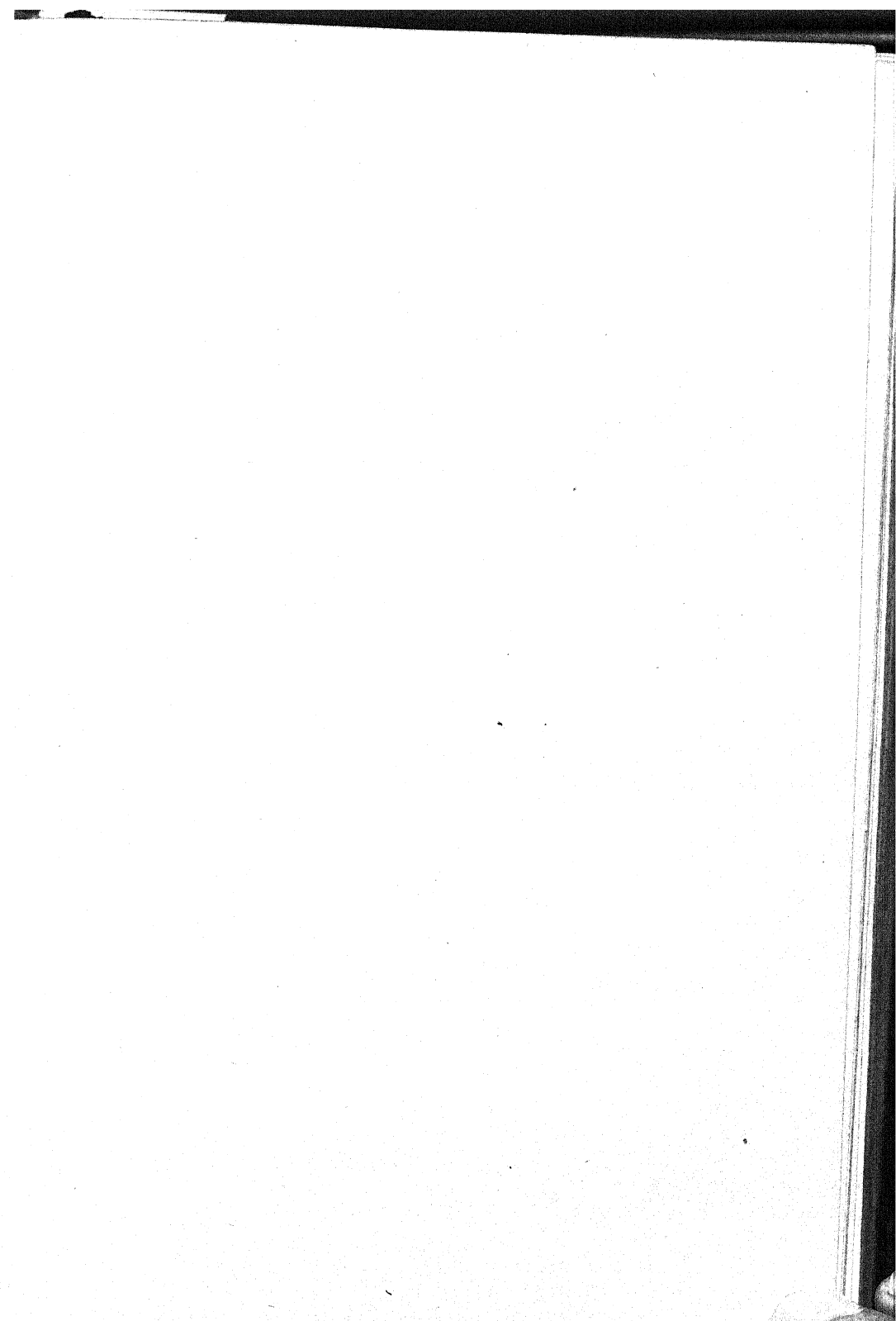
The author is indebted to his colleagues for constructive discussions of this subject; to W. R. Meagher and G. Garbutt, who also skillfully drafted the figures; to my parents and wife, my faithful and constant helpmates.

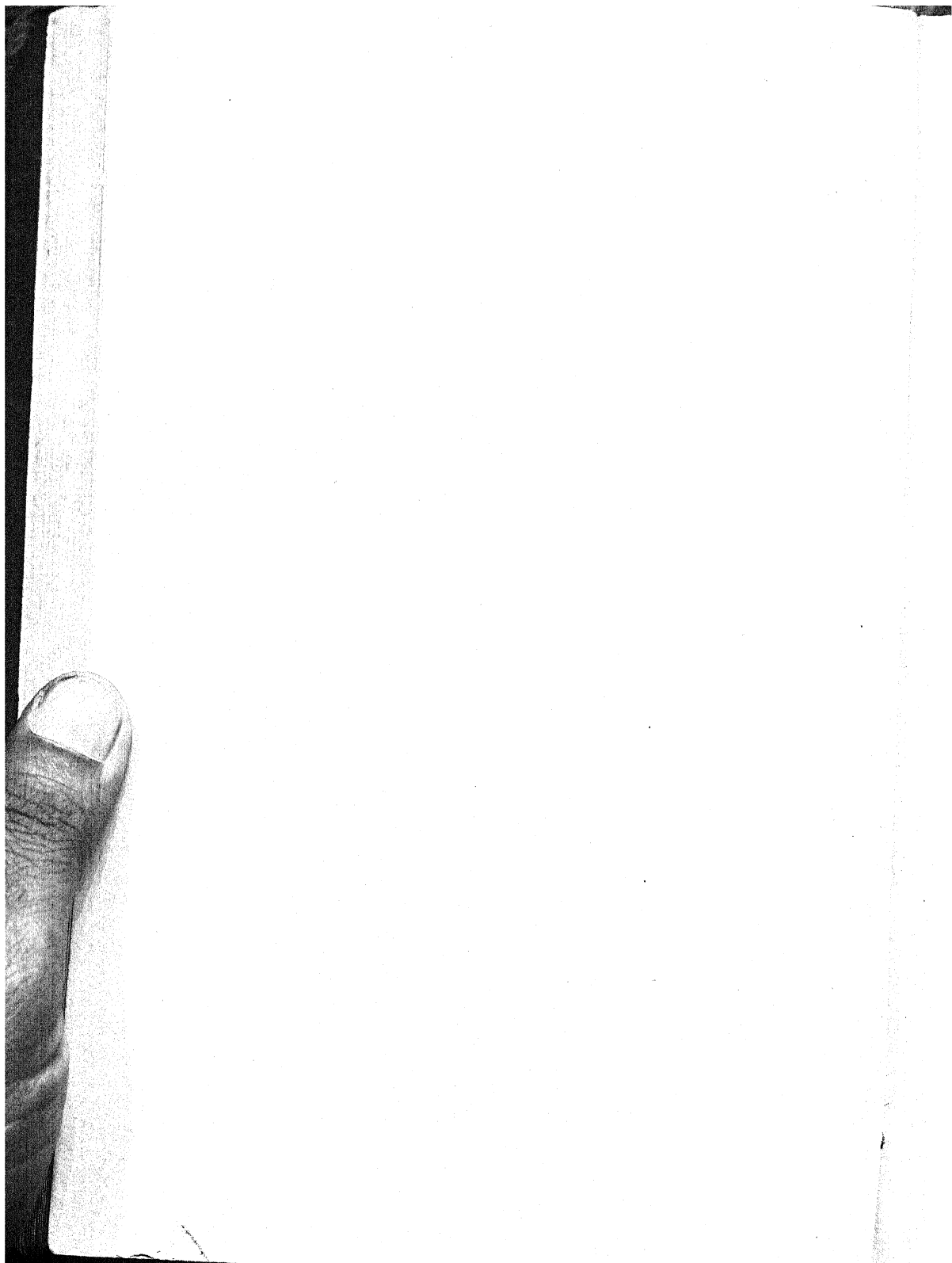
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ANTIBIOTIC PRODUCTS OF FUNGI

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It has been recognized from the earliest days in the development of the science of microbiology that certain microorganisms, gaining access to pure cultures of bacteria as contaminants, may suppress the growth of a species one is desirous of cultivating. It is probable that every microbiologist has seen, at some time in his career, plate cultures of bacteria and mold wherein wide zones free of growth surround certain colonies. Such colony growths tend to distinguish themselves through the secretion of substances, commonly called antibiotics, that prevent the encroachment of other species of microbic life. It would appear that the significance of natural antagonisms such as these might long ago have been recognized had not the microbiologist in his intensive search for deeply hidden mysteries ignored the plainly visible phenomena and literally failed to see the forest for the trees. It remained for Fleming, therefore, to appreciate fully the significance of this exhibition of microbial antagonism and to investigate its possibilities.

The microorganism that first attracted Fleming's attention was a mold of the genus *Penicillium* which had contaminated a staphylococcus plate culture and caused the bacterial colonies in the immediate vicinity of the contaminating fungus to become transparent and show other evidences of undergoing disintegration. Subsequent experiments demonstrated that the contaminating mold could be grown in fluid culture media and that filtrates of these broth cultures contained substances of exceedingly potent antibacterial nature for a wide variety of Gram-positive organisms. The mold itself was considered by Fleming to be a strain of *Penicillium rubrum*. It was not correctly identified until 1932 when Thom examined a culture and reported that he believed it to be *Penicillium notatum* Westling in the *P. chrysogenum* Thom series.

The antagonistic principle of the mold was designated by Fleming as penicillin. He did not attempt its extraction from crude broth filtrates but, nevertheless, suggested its potential value in the treatment of infections resulting from penicillin-sensitive microorganisms. He also made considerable use of penicillin in culture media as an agent for suppressing growth of Gram-positive contaminants in the isolation of *Hemophilus influenzae* from sputum.

Although Fleming so ably demonstrated in 1929 that broth filtrates of *P. notatum*, even when diluted several hundred times, possessed a remarkable toxicity for certain species of bacteria and simultaneously were non-toxic to white blood corpuscles, his discovery failed to arouse widespread interest until 1940. In the meantime, however, Clutterbuck, Lovell and Raistrick had found that the mold could be grown successfully on synthetic media. Furthermore, these investigators had attempted ether extraction of the active principle but failed to obtain a purified substance in an active form. Between 1935 and 1940 two independent American investigators, Reid and Bornstein, had published results of their researches on the antibacterial activity of broth filtrates containing penicillin. Finally, a group of distinguished scientists under the leadership of Dr. Howard W. Florey, at the Sir William Dunn School of Pathology, Oxford, England, undertook the problem of purifying penicillin and determining its chemotherapeutic properties. The results of their very thorough investigation, published in 1940, aroused, as one writer has so aptly stated, such widespread and feverish research as practically to assume the dimensions of a scientific gold rush. The sudden interest in antibiotic products of mold growth undoubtedly was stimulated by the remarkable success being experienced by 1940 in the therapeutic use of sulfa drugs. The microbiologist was among the first to realize the significance of the sulfa drugs and, therefore, had no difficulty in foreseeing toxic products of living organisms themselves as powerful agents in the treatment of infectious diseases. One could readily deduce that, if the antibiotic substance is harmless to the cell secreting it, this agent might likewise be non-toxic to certain other cells, such as those of higher plant and animal life.

Most of the emphasis in the investigation of antibiotic products of mold has been placed on *P. notatum*, which is now known to

yield not only penicillin but other growth products, called variously, "chrysogenin" (131) and "notatin" (31), "penatin" (91) or "penicillin B" (123, 141), possessing varying degrees of toxicity for bacteria. Additional species of the genus *Penicillium* have been investigated and found to yield antibiotic substances. These are *P. puberulum*, shown as early as 1913 to produce penicillic acid (113); *P. citrinum* and *P. cyclopium*, investigated more recently (112, 113) and found to produce citrinin and penicillic acid, respectively, both of which possess some antibiotic properties; *P. claviforme*, producing a substance called "claviformin" (21); and *P. patulum*, elaborating an agent designated as "patulin" (117). Some of these agents have been described as being effective against both Gram-positive and Gram-negative organisms.

In 1943 Waksman and his investigators described an antibiotic from *Aspergillus fumigatus* which they called "fumigacin". This substance has been shown to be the same as one designated by another group of investigators as "helvolic acid" (22). *Aspergillus flavus* has been examined for antibiotics by various workers and found to produce substances variously named as "aspergillic acid" (159), "flavicin" and "flavicipin" (16, 106). These agents have been shown to behave like penicillin and are probably identical to it. Another substance said to be similar to penicillin is "gigantic acid" derived from *Aspergillus giganteus* (116). Waksman's (144) thorough examination of "clavicipin" (150) and "clavatin" (9), antibiotics from *Aspergillus clavatus*; "claviformin" from *P. claviforme*; and "patulin" from *P. patulum*, has shown these agents to be identical in their chemical nature and antibacterial activities. Thus, we have evidence that at least three different species of mold produce the same bacteriotoxic substance.

Much confusion has arisen as a result of the ability of a variety of molds to engender identical antibiotic agents which, considered by their discoverers to be unique, have been assigned special names to differentiate them from the very substances with which they have later been proved identical. Such confusion is certain to occur in a field experiencing the feverish activity that has been taking place in the realm of antibiotics. We know now (138) that various molds, as indicated in the preceding paragraph, are capable of producing the same types of antibiotic substances. Thus penicillin is

formed by *P. notatum*, *P. chrysogenum* and *A. flavus*; penicillic acid by *P. puberulum* and *P. cyclopium*; citrinin by *P. citrinum* and *A. candidus*; gliotoxin by *Gliocladium* (108), *Trichoderma* (154) and *A. fumigatus*; clavacin by *P. claviforme*, *P. patulum* and *A. clavatus*; and spinulosin by *P. spinulosum* and *A. fumigatus*.

In 1943 Waksman and Hornung (149) isolated and studied 160 cultures of fungi from various soils and composts. Many of these fungi when streaked on agar plates previously seeded with bacteria exhibited antibiotic action, but they failed to produce soluble active substances when grown in liquid culture media. These investigators suggested at the time that this discrepancy may have been due to the fact that the fungi produced antagonistic effects only in the presence of the living antagonists, or that variable conditions for the development of antibiotic substances were not attained by the methods employed. Of the 160 fungi studied, it was possible to formulate nine distinct groups. These groups were established as follows: the *Chaetomium* Group; the *Aspergillus fumigatus* Group; the *Aspergillus clavatus*-*A. glaucus* Group; the *Aspergillus flavus* Group; the *Penicillium luteum*-*P. purpurogenum* Group; the *Fusarium*-*Cephalosporium* Group; and, finally, a miscellaneous group comprising some species of *Mucor* and *Rhizopus*. A few of the fungi placed in the miscellaneous group showed evidences of antagonism on agar plate cultures but exhibited no ability to elaborate antibiotics in fluid media. More recently, Waksman and Bugie (146) have investigated the *Chaetomium* group more intensively and have reported the discovery of an antibiotic from *C. cochliodes* to which they have assigned the name "chaetomin". This agent has been found to be active principally against Gram-positive bacteria.

From the foregoing paragraphs it is obvious that only a relatively few different antibiotic agents have been derived from the investigation of a large number of mold species. Furthermore, it is conceivable that purification of antibiotics at present regarded as unique will show some of these agents to be the same chemically as other antagonistic substances. Thus the total number of antibiotics now recognized may be reduced eventually to a relatively small number. Before actual purification and determination of chemical structure is accomplished, however, the literature will probably become further confused with observations on additional fungi,

wherein newly discovered antibiotics will be assigned specific names until they are finally identified as products already known to science.

In view of the extensive work done on penicillin and the remarkable nature of this antibiotic as regards its relative lack of toxicity for higher forms of life, while exerting a highly toxic effect on many kinds of microorganisms, a considerable portion of this review will be given to an examination of the voluminous literature that has accumulated on this particular antagonistic agent.

PREVALENCE AND GENERAL CHARACTERISTICS OF *Penicillium notatum* WESTLING

The members of the genus *Penicillium* are exceedingly prevalent in nature, and a number of species have been widely recognized as associated with spoilage of fruits and other foods. Also, at least two species have been of great importance to the dairy industry in the ripening of certain cheeses. Of the members of this genus capable of elaborating antibiotics, *P. notatum* Westling is extremely common and, therefore, a frequent contaminant of cultures in the microbiologist's laboratory. It belongs to the largest of the three main divisions of *Penicillium*, the *Asymmetrica*, and is placed in the sub-section *Radiata* along with *P. chrysogenum* Thom. *Radiata* is composed of species having the typical growth habit of forming broadly spreading colonies, radiating evenly in all directions from the center with an approximately circular outline.

The mold grows readily on common nutrient media, either solid or liquid, and develops profusely in the presence of sugars. Also, it grows well on synthetic media composed primarily of mineral salts and glucose or brown sugar. It has been isolated from a great many sources, different strains exhibiting slight cultural or microscopical variations, but all showing definite evidences of a close relationship. Two strains of *P. notatum* have come to be widely used in the production of penicillin. One is designated as NRRL 1249.B21 and is a derivative of Fleming's original strain. The other is known as NRRL 832 and comes from the culture collection of the Northern Regional Research Laboratory, Peoria, Illinois. The latter is an interesting strain in that it grows and produces the greatest yields of penicillin when submerged in a fluid culture medium as contrasted with strain NRRL 1249.B21 which reacts most favorably in surface culture.

CULTURE MEDIA FOR PENICILLIN PRODUCTION

Many different nutritive substances have been used for growing molds for production of antibiotics. A considerable number of these have merely been modifications of media previously in use and have been claimed by their discoverers to produce increased yields of the antibiotic. Flenning originally employed a trypsin digest broth and considered it the best medium in which to grow *P. notatum*. Later (3) it was found that Clutterbuck, Lovell and Raistrick's (26) modification of synthetic Czapek-Dox medium was a better nutriment. This medium contains sodium nitrate, 3 gm.; potassium acid phosphate, 1 gm.; potassium chloride, 0.5 gm.; magnesium sulfate, 0.5 gm.; ferrous sulfate, 0.01 gm.; and glucose, 40 gm. per liter of distilled water. One of the early modifications of this formula was made by McKee and Rake (105) who substituted 40 grams of brown sugar for the glucose. Subsequently (76) it was recommended that the amount of brown sugar be reduced to 20 grams. Foster and his associates (54) concurred in the opinion that the substitution of brown sugar produced a superior medium and found that an additional improvement could be made by doubling the amount of sodium nitrate. The presence of iron salts was likewise found (149) to be favorable to the development of antibiotics, whereas manganese was said to have little effect. Others have shown that ferrous sulfate and potassium chloride can be eliminated and the amount of potassium acid phosphate reduced without affecting penicillin production. However, the phosphates may be significant as buffering agents, according to Challinor (24). Waksman and Hornung reported zinc to be quite detrimental, even in low concentrations of 10 mg. per liter. However, Foster and his associates later demonstrated that zinc in concentrations of 1 to 3 mg. per liter is an important element in promoting growth of the mold and in increasing yields of penicillin. They assumed zinc to act as a catalyst to the oxidation and use of glucose by the mold, thus preventing the accumulation of gluconic acid which plays an important rôle in lowering the pH of the medium. Recently (89) citric acid, borax and boric acid have been reported as stimulating to penicillin production in submerged culture.

Taylor (137), employing the Czapek-Dox formula as a base, tested the influence of addition of various sugars to the medium on penicillin production. The addition of sugar caused the medium to

remain acid for a longer period as growth progressed and also caused an increase in the amount of mold growth. The sugars, each of which was employed separately, were glucose, sucrose, maltose and lactose. Of these, 5% concentrations of the sugars produced the highest yields of antibiotic. When lactose was used, the antibiotic did not disappear quickly after attaining its maximal concentration, as is generally the case, but remained in the medium at a fairly high level for as long as 30 days. The lactose added to Czapek-Dox medium containing glucose apparently did not enhance the yield of antibiotic, but tended to preserve it, once formed. Early studies suggested that lactose was not utilized. However, recent reports (93) indicate that this sugar is oxidized at a certain stage in the growth of the mold.

Addition of 2% corn oil to a modified Czapek-Dox brown sugar medium (80) has been found to induce increased yields of penicillin from mold culture, and, likewise, to exhibit some preservative effect. Corn oil has been employed in certain commercial processes to prevent foaming in aerated, submerged cultures, rather than as a possible carbon source for the mold.

Most of the available information on the metabolism of *P. notatum* on various media appears to have been incidental to the more pressing problem during the war years of producing penicillin quickly and in quantity. One of the most important discoveries in the accomplishment of this objective was made by Moyer of the Northern Regional Research Laboratory, Peoria, Illinois, who found that addition of corn steep liquor to the nutrient medium used for mold cultivation increased the yield of penicillin tenfold. Later improvements in the corn steep liquor medium resulted in still greater yields. Thus, whereas the Oxford workers had originally obtained no more than two units per ml. it was now possible to obtain as much as 200 units per ml. Corn steep liquor is a valuable nutritive material that was earlier used as a nitrogen and mineral nutrient for yeast production and was known to the trade as "yeast compound". It is primarily an extract of corn solubles under acid conditions of pH 4-4.5. Dilute sulfurous acid and lactic acid play the principal rôle in the extraction. Laboratory and plant study has shown that during processing an active microbial population of lactic acid bacteria and yeasts assists in the extraction. Corn steep liquor is high in amino acids and minerals and in most of the B

complex vitamins. Its value in antibiotic production is in part due to the extensive fermentation it undergoes during the wet corn milling process (98).

Incorporation of dextrin in corn steep liquor has been stated (115) to yield equally as high a penicillin content in mold culture as when lactose is used. In other words, dextrin appears to provide the same preservative action as lactose and is more readily obtainable and economical. Sulfite waste liquor (115), a by-product of the paper industry, has also been employed as a base in the preparation of nutrient media for molds. Sugars are added to the liquor base.

Comparative studies (152) on growth and penicillin production by a number of strains and species of the genus *Penicillium* in Czapek-Dox, brown sugar, corn steep and corn steep-zinc media have shown that mold growth in the latter invariably produces greater yields of antibiotic. Certain strains of mold will, of course, elaborate a greater abundance of penicillin in one medium than in another. Cottonseed meal (55) is at least as good as corn steep liquor for penicillin production in submerged culture by *P. chrysogenum* strains Demerec X1612 and Wisconsin Q176. Without added chemical precursors, cottonseed meal is said to be considerably superior to corn steep liquor.

Radioactive substances have been reported (82) to activate the growth of *P. notatum*, thereby decreasing the time required to obtain active penicillin. The observed effect is presumably due to Alpha particles. Production of penicillin in the presence of Beta radiation has been investigated with both surface and submerged cultures (83). The radiation source has been radioactive phosphorus, P^{32} , in the form of phosphoric acid added to the production media before inoculation to give activities ranging between 0 and 100 microcuries per ml. of medium. The presence of beta radiation in the medium has tended to interfere with penicillin production, particularly in submerged cultures.

Bombardment of spore suspensions of *P. notatum* (64) with fast and slow neutrons has resulted in the formation of numerous interesting variations. The variant strains have differed greatly in their capacity to produce penicillin. Some no longer have this ability, whereas others have given yields considerably higher than those obtained from the parent culture. No qualitative differences

in the inhibitory substance produced by the variants have been recognized.

A medium consisting of an enzyme digest of purified casein and pork pancreas in which the proteins have undergone hydrolysis to amino acids, dipeptides and tripeptides, and known commercially as "amigen", is said to be very satisfactory for the growth of *P. notatum* (137). Also, a simple medium of tryptone, glucose and sodium chloride has been reported to promote considerable growth (39).

The natural tendency of the *Penicillia* to develop on fruits and vegetables has led to the extraction of various vegetables (30) as media for growth of *P. notatum*. Of the vegetables used, peas and pea flour have given the most satisfactory results. Maximum yields of penicillin in these media have been attained at 22° C. in 8 to 10 days, after which the media become too alkaline to preserve the antibiotic substance.

Studies on the control of the pH of *P. notatum* cultures through their carbon and nitrogen nutrition have been reported (36). It was found that the pH can be well regulated by supplying the mold with appropriate sources of carbon and nitrogen. Sources of carbon were glucose, sucrose, lactose, galactose and maltose. Sources of nitrogen were sodium nitrate, tryptophane, asparagin and cystine. When nitrate was supplied during a 12-day incubation period, the pH first dropped slightly and then rose to the range favorable to penicillin production, regardless of the sources of carbon. The medium containing glucose became practically neutral after 12 days, while that containing maltose remained at pH 5.5. In the presence of amino acids, the relation of pH to time of incubation varied with the sugar. When lactose was supplied, the pH remained practically neutral throughout a 10-day period, whereas with maltose and fructose it dropped slightly to pH 6 and then remained constant. When the other sugars, used as carbon sources, were employed separately or in mixture, the pH fell to a range of 3.5 to 4.5 and remained there until autolysis began. The pH also fell somewhat in media containing both amino acids and sodium nitrate, but not as markedly as when either of these nitrogen sources was used alone. After the initial fall it rose to a level intermediate between that attained with nitrate alone or amino acids alone. Thus it appears that incorporation of graded quantities of nitrate and amino acids

in media makes possible the attainment of any desired pH for a particular stage of the growth period when a carbohydrate is employed that is readily metabolized to organic acid.

The initial drop in pH and subsequent rise has been ascribed to the formation of gluconic acid from glucose or sucrose (54), with the trend back toward neutrality being due to the utilization of the nitrate ion without a corresponding use of the sodium ion. The latter is assumed to become associated with hydroxyl ion from the water in the medium and to result in a gradual pH rise. Nitrate metabolism in higher plants has also shown this trend of pH with time (111). Dimond and Peltier suggest the trend toward decreasing pH values with amino acids as probably due to an accumulation of organic acids resulting from deamination of amino acids as well as from formation of gluconic acid when glucose is the source of carbon. As the glucose concentration is reduced, the organic acids resulting from amino acid metabolism may be used more readily as a source of carbon than the sugar, causing the pH to remain practically stationary. The use of amino acid derivatives as sources of carbon by the fungus may also explain growth in media containing sugars not readily fermented by the mold (if at all), such as lactose. Regulation of the pH of culture media by supplying the mold with appropriate sources of carbon and nitrogen appear to have definite value in the production of penicillin, since the pH of the nutriment must be kept in the range of 5.5 to 7.5 for maximum production (3).

A recent report (136) states that most amino acids fail to induce increases in penicillin. l-leucine and certain of the sulfur-bearing amino acids have occasionally given increased yields, but the effect has not been consistent. A series of aromatic compounds related to phenylacetic acid and phenylethylamine have markedly stimulated penicillin yields. Phenoxyacetic acid and the meta- and para-halogen derivatives of phenylacetic acid have been quite effective. In most cases the amide derivatives have been just as satisfactory as or better than the corresponding acids. Whether sugars have been employed with these compounds as sources of carbon is not clear.

METHODS OF CULTIVATION

All antibiotics known at present are derived from microorganisms requiring atmospheric oxygen in their metabolic processes. Three

methods of cultivating antibiotic fungi have been developed. One consists of shallow surface culture, wherein spores distributed over the surface of the fluid medium are permitted to develop into a floating, leathery mat. Another consists of shallow submerged culture, in which the organisms are inoculated into a thin layer of liquid, and air reaches the lower portion of the fluid by diffusion. A third method employs deep submerged culture, wherein the organisms can grow at any depth due to the forcing of air through the fluid medium.

Early studies on the production of penicillin employed the surface culture technique. The mold spores are inoculated onto the surface of a fluid nutrient medium which is generally less than 2 cm. in depth. Almost any type of glass container with a mouth large enough to permit ready interchange of air can be used in cultivation of the mold. Metal containers have been found to be unsatisfactory unless enamel coated, since heavy metals tend to exert a destructive action on penicillin.

Penicillium notatum develops and elaborates its antibiotic substance most readily at 24° C., although it will grow quite satisfactorily at temperatures between 22° and 25° C. At higher temperatures, sporulation fails to occur. The mold will not develop at 37° C., the most favorable temperature for bacteria pathogenic to man. When the spores are inoculated into a thin layer of fluid media, a delicate, fluffy, gauze-like growth is faintly visible after 24 hours. The surface growth generally develops first in isolated foci, especially around the sides of the culture flask, where it presents a dry, whitish appearance. Usually after four or five days of incubation at 24° C. the entire surface of the medium is covered with dry mycelium which turns bluish-green within about 24 hours after it appears. This is the result of sporulation. The color is at first centralized with a surrounding zone of white mycelium, but by the end of a week of growth the surface is covered with a heavy corrugated layer of bluish-green felt. The under side of this layer is golden or yellowish and slimy. It is freely wetted with water in contrast to the upper surface which can not be wetted. Droplets of a yellowish chrysogenin frequently appear on the upper surface and collect in the corrugations of the felt-like layer, but do not wet it. As incubation of broth cultures is prolonged, the blue-green color of the surface fades and may eventually become a dull gray.

The pH of media supporting surface cultures usually drops to about 3.5 to 4.5, and remains practically unchanged during the first three or four days after inoculation. Then as the mold growth becomes more profuse, the pH tends to rise. The increase in pH is accompanied by development of antibiotic activity that generally reaches its maximal strength when the medium approaches neutrality. As the pH rises beyond the neutral point, the medium rapidly loses its antibiotic effect. Generally the medium is found to exhibit its maximal antibiosis at the time numerous droplets of chrysogenin appear on the surface. This is usually seven to ten days after inoculation. The antibiotic effect is best preserved by filtering off the mold at this time and adjusting the pH of the filtrate to 6.5 to 7. If the crude filtrate is stored at refrigerator temperature, it may remain active for several weeks, as long as the pH is kept properly adjusted, or if frozen the antibiotic effects of the filtrate may be retained for several months.

Production of penicillin by submerged culture is now an important commercial method. However, early attempts to attain significant yields by this method were disappointing, even when mechanical agitation was used to prevent formation of surface mats and supply air to the submerged growth. Reid (122) had shown that strains of *P. notatum*, other than the one originally isolated by Fleming, demonstrated penicillin-like properties. Hence, investigators at the Northern Regional Research Laboratory isolated and examined hundreds of strains in an effort to find cultures capable of producing higher yields of penicillin than the original strain of Fleming. Among the strains of *Penicillium* isolated was one now designated as NRRL 832, which was a poor producer of penicillin in surface culture but which gave exceedingly large yields when grown in mechanically stirred, aerated, submerged culture tanks. According to Coghill, the principal problem involved in the submerged culture process is whether the strain of mold employed will grow beneath the surface of the nutrient medium and thereby make possible the use of huge tanks or vats for large scale production of antibiotics. Since the submerged growth requires aeration, mechanical agitation and air dispersal is used. The mold, thus supplied with air, grows in submerged culture in the form of small pellets rather than as a heavy mat.

According to Koffler and his associates (93), the carbon dioxide

level seems to have greater influence on penicillin production in submerged fermentations than the pO_2 of the culture atmosphere. Strains growing well in surface culture are not suitable for submerged culture processes. Coghill pointed out that yields of penicillin as high as 80 units per cubic centimeter could be obtained in two days through submerged culture, and, more recently, improvements in the deep culture process have resulted in yields as high as 250 units or more per cubic centimeter. Contamination of a large scale deep culture system can quickly result in the total loss of a batch of penicillin. Therefore, extreme care must be taken to prevent the entrance of other forms of microbic life. Borax and boric acid, when used at a level high enough to delay the growth of contaminants and still not interfere with penicillin production, may be of value in the prevention of extraneous growths (89).

In addition to the surface and submerged culture methods, others have been described. For example, Clifton (25) described a method whereby continuous production of penicillin was effected by means of a culture solution of yeast extract or corn steep liquor trickling through a column of wood shavings supporting a growth of *P. notatum*. The solution drained off below contained antibiotic properties. This method, similar to the quick process for making vinegar, was adjudged difficult to employ on a large scale because of the ease with which the system might become contaminated. However, Clifton reported in his preliminary work that one operation continued satisfactorily without contamination for 15 days. Another type of drip method (129) consists of inoculating the mold culture on a solid agar base containing constituents favoring a rapid production of penicillin in drops of sufficient size and amount to be precipitated on the opposite side of the container, when inverted. Clear drops of penicillin form after five or six days and may be withdrawn by means of sterile pipettes. This method was described as being suitable for producing penicillin for small-scale topical application.

Penicillin can also be produced by growing suitable strains of *P. notatum* on moist bran which is thinly spread in a shallow tray or processed in a rotary drum. Each particle of bran offers its entire surface as a substrate for growth. The bran must be sterilized before inoculation with the culture. Yields of as much as 200 to 400 units of penicillin per gram of dried bran have been obtained

after several days of growth by this method. Coghill (27) states that the difficulties of this process are that bran is a very poor conductor of heat and can be sterilized only with considerable difficulty. Hence, contamination occurs readily. Secondly, although the temperature employed for incubation of the inoculated bran is approximately 24° C., a considerable amount of heat is produced during fermentation and must be dissipated by some means to prevent too great a rise in temperature (37).

Use of the bran culture process has been limited by technical difficulties, but it has come to be quite a satisfactory method for production of mold spores to be employed as seed stock for inoculation of surface and submerged cultures on a large scale. A considerable yield of spores for seed may be obtained by use of a relatively small amount of bran. In one method (132) 30 grams of large wheat bran particles are placed in 750-cc. conical flasks. The bran is thoroughly moistened with an equal weight of water and the flasks are sterilized for one hour under 15 pounds of steam pressure. After sterilization each flask is inoculated with 1 ml. of a spore suspension of *P. notatum*, shaken and incubated at 24° C. for two days. This method serves both as a means of obtaining a potent crude extract of antibiotic for local application to surface wounds and of spores that can be preserved for seeding subsequent culture media.

RECOVERY OF PENICILLIN FROM CULTURE MEDIA

The mycelium of the mold can be readily removed from the culture medium by filtration. However, the crude filtrate was early recognized to contain pyrogenic, or fever-producing, substances, and it therefore could not be used safely as a therapeutic agent, except in local applications on surface wounds. Hence, the Oxford workers (1941) made a study of methods that might be successfully employed to extract the antibiotic agent in a purified form. It was found that solvents, such as ether, amyl acetate and ethyl acetate, could be used for extraction of penicillin from broth filtrates. This is done most readily by adjusting the pH to 2 or 3, using as low a temperature as possible and extracting rapidly, since penicillin is very unstable at the low pH. One method employs ethyl acetate (30) as the solvent after acidification of the medium with phosphoric acid. More recently (10) a solvent has been introduced

that will accomplish the extraction at a pH not harmful to penicillin. This solvent is n-butyl alcohol which will take up a large portion of the penicillin at pH 6.4. The stability of the antibiotic is not affected at this pH. According to Berger, addition of ammonium sulfate aids precipitation of the greater part of the inactive pigments and also accomplishes almost complete extraction of penicillin. After extraction with n-butyl alcohol, the penicillin can be brought back into aqueous solution by addition of light petroleum ether and shaking the mixture into dilute sodium bicarbonate solution. The advantages claimed for this method are almost complete recovery of penicillin at a pH harmless to the antibiotic with relatively small amounts of solvent, and extraction at room temperature.

Crude culture filtrates containing penicillin, when passed through cationic resin, Ionae C, at pH 6 and 7, followed by passage through anionic resin, Ionae A, at the same pH, become free of toxicity for experimental animals and man, yet retain all the penicillin activity of the original material. The penicillin preparation thus obtained can be concentrated *in vacuo* at low temperature (33).

Commercial methods for penicillin production undoubtedly vary slightly from one another. However, in one commercial process for preparation and extraction of penicillin, the mold spores used as seed stock are stored in soil below 0° C. Agar slants are prepared from the soil cultures and, after growth has taken place, transplants are made to moist bran. Spore suspensions obtained from the bran are transferred to small batches of nutrient media which are used later to inoculate larger tanks of the medium, known as seed tanks. Huge vats or tanks are in turn inoculated from the culture grown in the seed tank. After a suitable growth period, the nutrient medium containing the antibiotic is separated from the mold by means of a Bird-Young filter. The filtrate is then retained in large adsorption tanks. The adsorbed penicillin is filtered and eluted with acetone, after which the acetone is recovered. Next the penicillin is taken up in a solvent, centrifuged and finally clarified by filtration. A sodium or calcium salt of penicillin is then prepared, sterilized, dried in large vacuum driers and sealed in proper amounts in ampoules. Before distribution the salt is tested for freedom from fever-producing substances, sterility, potency, moisture and toxicity (99).

Regardless of the solvent used, it is important that penicillin be

rapidly converted from the organic acid state in which it exists in the broth culture to an organic salt after extraction. The sodium salt of penicillin is the one that has been most commonly prepared. This salt is obtained by adding sodium bicarbonate to the solvent containing the extracted penicillin and shaking the mixture. When ether or amyl acetate are used as solvents and the broth filtrate is reduced to pH 2 for extraction, the high acidity must be quickly readjusted to pH 6.5 to 7 immediately after the penicillin is taken up in the solvent, otherwise great damage will be done to it. Sodium penicillin is hygroscopic, is relatively unstable in water and is destroyed by heat. Hence, it is dried by a lyophilizing process similar to that used for preparing dried blood plasma. The final product is a yellow, amber or brown powder. The variation in color is due to the fact that it is not a pure product, but contains some of the organic substances that were present in the medium in which the mold was cultured. Failure to obtain an absolutely pure product has interfered with the study of the chemistry of penicillin.

A recent innovation in the drying of penicillin has been the use of radio heat (14) wherein the product is said to be properly dehydrated 48 times as rapidly as by the freeze-drying procedure. This electronic method is considered to be extremely economical.

DIFFICULTIES ENCOUNTERED IN PENICILLIN PRODUCTION

In the production of penicillin by surface culture it is sometimes difficult to obtain a uniform inoculation and to produce a continuous growth of mold over the entire surface of the medium. Hence, seeding of culture flasks with spore suspensions in corn oil (80) and in a medium containing gum tragacanth and an emulsion of lanolin (153) have been suggested. An ingenious method for increasing surface areas of both surface and submerged flask cultures of the mold has been introduced. This consists of the use of cellophane bags or strips which permit the growth to extend well above the surface (128). Finely ground cork has also been used. Greatly increased yields have been obtained. Even degenerate cultures respond well.

In spite of all the numerous aids to production of greater yields of penicillin, one of the most important factors is the strain of mold itself. Strains of *P. notatum* differ markedly in penicillin-producing ability. Furthermore, active strains tend to lose their capacity to produce penicillin, especially after long serial transfer on labora-

tory media (54). This degeneration of *P. notatum* has also been noted in connection with the formation of the yellow pigment, chrysogenin (26). Degeneration may take place without conspicuous cultural changes, although degenerate cultures often fail to sporulate readily. Cultures allowed to incubate on agar surfaces for a period of time, beyond that necessary for maximal sporulation, may show secondary developments of white, cottony patches that gradually spread over the surface. Cultures of this type usually yield subcultures weak in penicillin activity.

P. notatum is considered a form species rather than a true species (100). In nature many conidia germinating together may anastomose if the strains are compatible and immediately produce heterokaryons. In the laboratory, however, single conidia germinate on solid media separately. Hence, cultures are selected that have originated from single conidia. Many are homokaryotic. This is particularly true of fungi, like *Penicillium*, with uninuclear conidia. Lindegren and Andrews point out that the high lability of penicillin production suggests that in nature, where mixtures are the rule, little or no penicillin is produced. Only in the laboratory where homokaryons are isolated, or where a single spore falls on an agar plate as in Fleming's original observation, can appreciable amounts of the antibiotic be expected. Nevertheless, the possibility of increasing yields by making mixtures has been suggested. Possibly cytoplasmic hybrids of *P. notatum*, producing far greater yields of antibiotic than any now known, will eventually be developed.

Frequent, successive transfers of the mold on artificial media contribute to degenerative changes. This, of course, is true of microorganisms in general. It has been suggested (54) that each culture may be composed of a mixture of substrains or cells heterogeneous in regard to penicillin production. During continued propagation the poor substrains may overgrow the good strains and predominate in the culture. Single spore or colony isolations from a given parent culture show marked variation in penicillin-producing activity. Generally the tendency is toward a reduction, but isolates equal to or even better than the parent strain may be found. The degeneration of penicillin-producing activity can be prevented by reducing vegetative transfers of stock cultures. Cultures of high activity should be selected and the spores from them mixed in several tubes of dry sterile sand or soil followed by drying at low

temperature. Spores retested for activity immediately after the drying process (54) have been found to be as satisfactory as the original culture. The dried cultures serve as the master stock cultures and maintain full penicillin-producing activity indefinitely. In the preparation of these cultures it is important to stop incubation within one day after maximal sporulation takes place which is usually three or four days after the beginning of incubation.

A single spore analysis of a stock culture of *P. notatum* (63), according to the method of Hansen and Smith (62), has shown it to be a dual fungus, composed of two distinct constituents associated together in culture. This dual phenomenon (61) has been found to be characteristic of most if not all fungi. The two components of *P. notatum* are described as a normal conidial or C type and an abnormal or M type. The M type appears repeatedly as a mutant of physiologically aging colonies of C type originated from a single conidium.

The M type is both morphologically and physiologically distinct from the C type and tends to predominate where mass transfers of inoculum are used. Pure cultures of the C type, maintained in a state of physiological youth by frequent transfer, tend to remain free of M type. The M type tends to be non-sporulating and pigment-producing and is probably predominant in cultures described by various workers as poor penicillin producers. Baker (8) has suggested that from types C and M, single conidia could comprise either of these genetic factors or their combinations—C, M, CM, CC or MM—depending on the number of nuclei per conidium. Heterotypic spores would possess the genetic means of variation from the outset, whereas homotypic spores would presumably develop monotypically, provided no mutations occur. Anastomosis among developing germ tubes, conidia and mycelia occurs shortly after germination of spores transferred in mass, giving opportunity for nuclear interchange. Hence, mass transfer of spores has been suggested as a means of increasing the chances of nuclear mixing and of heterokaryotic vigor in cultures.

PHYSICAL AND CHEMICAL PROPERTIES OF PENICILLIN

Penicillin exists in crude culture filtrates as a strongly dibasic organic acid that is very unstable (10), and loses its activity quickly on exposure to air or to heat or at a pH below 5 or above 7. Also,

certain bacteria, as contaminants in a culture, can destroy the antibiotic. Free penicillin (2) is readily soluble in alcohol, ether, acetone, ethyl and amyl acetate, cyclohexanon and dioxane. It is only partially soluble in benzene, chloroform and carbon tetrachloride. It will dissolve to the extent of 5 mg. per ml. in water (19). It is very sensitive to acids, alkalies, primary alcohols, oxidizing agents and heavy metals, but less so to reducing agents. Penicillin can be most readily preserved in the form of a salt, the sodium and calcium salts having been most thoroughly investigated (20, 59, 67). The calcium salt is not hygroscopic like the sodium salt and can be preserved for longer periods of time. The barium salt, a non-hygroscopic white powder, which is stable in a dry state for a long period of time, has been prepared (2), as has a yellow strontium salt (17) and an ammonium salt (76, 109). Like sodium salt, the latter is very hygroscopic. Various esters have been prepared by Meyer and his co-workers (110). Esters are found to be insoluble in neutral or slightly alkaline buffers, but very soluble in benzene. They have shown considerable activity *in vivo*, are not destroyed by gastric juices and can be administered by mouth. The empirical formula of the barium salt (2) has been given as $C_{24}H_{32}O_{10}N_2Ba$ or $C_{23}H_{30}O_9N_2Ba$. Evidence indicates one ketonic, 2 acetyl stable and one latent carboxylic group. Catch *et al.* (17) found the empirical formula of the strontium salt to be $C_{26}H_{34}O_{11}NSr$. Meyer (109) gives the formula for the ammonium salt as $C_{14}H_{19}NO_6$ or as $C_{14}H_{17}NO_5 + H_2O$. The latter was found to be strongly dextrorotatory and had an absorption maximum on spectrographic examination of 2750 Å. Much information concerning the molecular structure of penicillin has been attained through the use of infra-red spectra. On the basis of diffusion constants (56), the radius of the penicillin molecule has been determined as 5.37 Å. with a molecular weight of approximately 500.

DETERMINATION OF THE POTENCY OF PENICILLIN

Methods for determining the potency of penicillin are based on the bacteriostatic effect a sample exhibits for a given test organism. The bacteria commonly used in evaluating the strength of penicillin solutions are *Staphylococcus aureus* (H), a strain originally selected by the Oxford workers (3), and spore suspensions of *Bacillus subtilis* (52). The original method for determining the potency of

penicillin was one of serial dilution (40). This proved to be a laborious technique that could be applied only to sterile material. If the penicillin-bearing material to be tested was sterilized by filtration through Seitz filters, some of the antibiotic was apt to be absorbed on the filter pads, so the true potency of the material under filtration could not be ascertained. Hence, the principal methods now employed are the agar plate assay (3, 52), the turbidimetric method (50) and the tissue culture preparation (66). Of these, the agar plate or cylinder cup method is the one most widely used for routine purposes. The agar surface may be seeded with the test organism by allowing a broth culture to flow over it and draining off the excess broth, after which the plate is dried for an hour at 37° C. (3), or in the case of spore suspensions the melted agar may be seeded before it is apportioned into the plates (53). The penicillin under test is placed in small glass or unglazed porcelain cylinders set on the surface of the seeded agar. Or blotter discs may be saturated with the solution and placed on the agar (130). The potency of the penicillin determines the size of the zone of colony inhibition around the cylinder or disc after incubation. However, the extent of inhibition is meaningless unless a penicillin solution of known strength is used simultaneously as a control. The strength of a penicillin solution is expressed in terms of units. The unit was originated as an arbitrary value by the British investigators and is now frequently referred to as the Oxford unit. Once the unit was defined, it has been possible to maintain penicillin preparations of known unit value as standards and to determine the strength of an unknown by comparing it with the standard. Thus, the unit as defined by the British investigators is that amount of penicillin which, when dissolved in 1 ml. of water, gives the same inhibition of growth against a known inoculum of *Staph. aureus* H as the standard.

In 1944 an international conference on penicillin was called in London for the purpose of establishing an international standard for penicillin. In view of the fact that several different penicillin preparations have been made in England and the U. S., it was recommended that this International Standard be deposited with the Department of Biological Standards, National Institute for Medical Research, Hampstead, London, N. W. 3, from which point it could be dispensed to national control centers. Also, it was recom-

mended that the International Unit of Penicillin be defined as the specific penicillin activity contained in 0.6 microgram of the International Penicillin Standard (142).

Commercial penicillin since May 1944 has been less effective in the treatment of diseases, such as early syphilis, than that produced prior to this date. This trend of decreased efficacy coincides with the development of new strains of *Penicillium notatum* and *P. chrysogenum* for increasing the commercial output of the antibiotic. Actually, four penicillins, X, G, F and K, are known (28). It is now recognized that increased commercial output has resulted in the production of proportionately greater amounts of penicillin K at the expense of penicillin G, which predominated in the early commercial product. Penicillin K does not appear to have the therapeutic value of G in syphilis. It is important, therefore, that the amounts of effective and less effective penicillins in a given volume of the antibiotic be known. These can now be determined by partition chromatography (38).

MODE OF ACTION OF PENICILLIN

The statements appearing most commonly in the literature concerning the mode of action of penicillin suggest that its antibacterial effects are due to bacteriostatic action or ability to arrest growth as differentiated from bactericidal action or rapid destruction. This view advanced by various workers is supported by researches (50) suggesting that penicillin depends for its activity *in vitro* on the logarithmic prolongation of generation time. However, these investigations have employed turbidimetric data as a basis for estimating total numbers of bacterial cells and have not taken into account the probable accumulation of dead as well as living organisms. Other researches, based on actual numbers of viable cells (50, 51, 75) exposed to different concentrations of penicillin, show the agent to have definite bactericidal properties, the rate of killing being roughly an inverse linear function of the concentration of the antibiotic substance.

Gardner (57) observed microscopic changes in rod-shaped bacteria following inhibition of their growth in culture by penicillin. The morphologic variants appeared at incompletely inhibitory concentrations and were assumed to be due to failure of fission. The majority of cells occurred as unsegmented filaments, suggesting the

possibility that growth proceeded without division and separation following in due course. Penicillin *in vitro* appears to be active only against growing, susceptible organisms (76, 96). Bacteria in a state of physiological youth are quite resistant when kept in the cold where growth is arrested by the low temperature. Killing takes place when multiplication occurs, indicating that penicillin must interfere with processes involved in cell division. Lee *et al.* (96) have made studies suggesting that a fraction of each generation is killed in the presence of the antibiotic, the value of the fraction depending on factors such as the nature of the organism, the concentration of penicillin and the conditions of growth. It is said that the shorter the generation time the more destructive a given concentration of penicillin will be.

Electron photomicrographs show that penicillin does not cause lysis of treated bacteria as does bacteriophage, but that it inhibits or kills vulnerable organisms, following which certain cellular changes, including enlargement, may occur. In this respect the action of penicillin resembles that of gramicidin or that of the sulfonamides, in which visible cellular changes are less readily observed than in bacteriophagy (157).

Penicillin *in vivo* has been considered to aid the defensive mechanisms of the host in getting rid of invading organisms. However, the therapeutic value of single doses indicates that in some instances, at least, it must exert bactericidal action in addition to whatever bacteriostatic activity it may possess. There is also the possibility that the antibiotic may cause pathogenic bacteria to undergo dissociation within the host, resulting in loss of virulence. The finding of zygospor-like bodies in the urine of patients treated with penicillin has been described (4). These are morphologic variants of the common Gram-negative intestinal bacillus, *Escherichia coli*. The variants thus induced tend to revert to type on culture.

EFFECT OF PENICILLIN ON THE AGENTS OF INFECTIOUS DISEASES

Penicillin has proved to be far more effective against Gram-positive than against Gram-negative bacteria. This is shown by the results of studies conducted by a host of investigators who have performed both *in vitro* (73) and *in vivo* (45, 67) experiments. It has also been substantiated in the reports of physicians and veterinarians. There are a few Gram-negative organisms, how-

ever, that are notably susceptible to the action of penicillin. These include gonococcus, meningococcus and *Phytomonas tumefaciens*. The last is the cause of crown gall, a plant disease commonly regarded as incurable. However, application of crude penicillin has resulted in destruction of the galls (15). Other microorganisms susceptible to the action of this antibiotic are staphylococci, streptococci, some strains of micrococci, the pneumococcus, *Bacillus anthracis*, *Clostridium tetani*, *C. botulinum*, *C. perfringens*, *Corynebacterium diphtheriae*, *Actinomyces bovis*, *Streptobacillus moniliformis*, *Erysipelothrix rhusiopathiae* and *Treponema pallidum*. Organisms insusceptible to the action of penicillin include the Gram-negative Enterobacteriaceae, *Hemophilus influenzae*, *H. pertussis*, *Brucella melitensis*, *Vibrio comma*, *Mycobacterium tuberculosis*, *Blastomycetes* and *Monilia* (34, 35, 68).

Oral administration of penicillin over a period of months has shown striking changes in the microflora of the throat and intestinal tract (101). Prepenicillin throat cultures have shown a predominance of Gram-positive diplococci sensitive to penicillin, whereas Gram-negative organisms become predominant during the course of treatment. These even include coliform bacteria which are absent prior to therapy. Changes in the intestinal flora, although less striking, also occur. Nonhemolytic streptococci, recovered from stool specimens before administration of penicillin, have been found only infrequently during treatment. The literature on the chemotherapeutic value of penicillin has been fully covered up to 1945 (68).

AGENTS THAT INACTIVATE PENICILLIN

It is not uncommon for penicillin culture media to become contaminated with bacteria that cause inactivation of the antibiotic principle. Abraham and Chain (1) have shown that certain bacteria produce an extracellular enzyme capable of destroying penicillin. This enzyme is called "penicillinase". The resistance of some bacteria to the antibiotic has been ascribed to their ability to produce penicillinase. However, this supposition does not fully explain the tolerance that some bacteria eventually display as a result of prolonged exposure to penicillin. Bacteria most active in the destruction of penicillin are the Gram-positive, spore-bearing rods, *Bacillus cereus*, *B. megatherium*, *B. mesentericus* and *B. subtilis* (11, 161). In view of the fact that *B. subtilis* spore sus-

pensions are now commonly used in testing the potency of penicillin preparations, it is important that all vegetative cells be destroyed by heat in preparing the suspension, or there may be such marked accumulation of penicillinase during storage as to effect total failure in determining the strength of a given batch of penicillin. Other bacteria capable of inactivating penicillin are *Mycobacterium tuberculosis*, *Actinomyces lavendulae*, *Actinomyces antibioticus*, some species of *Pasteurella* and certain strains of *Staphylococcus*. Among the yeasts, *Mycoderma valida* and *Debaryomyces guilliermondi* are fairly effective in destroying penicillin. Molds appear to be relatively weak in penicillin-destroying powers. The production of penicillinase by microorganisms is affected by the presence of sugars and the pH of the medium in which the organism grows as well as by the incubation temperature. Certain enzyme poisons, such as sodium azide, iodoacetic acid and ferrous chloride, are inhibitory to penicillinase. Sulfhydryl groups (sodium thioglycollate) tend to activate the enzyme (161).

In view of the fact that penicillin preparations may become contaminated with organisms inactivated by the concentrated antibiotic, but capable of growing when the agent is diluted by the body fluids, it has been found advisable to introduce sterility tests on each lot of penicillin before its release. The preliminary step in these sterility tests consists of inactivation of the antibiotic so as to permit penicillin-sensitive organisms that may be present an opportunity to grow out in the medium. Clarase (94, 133) has been suggested as a penicillin-inactivator and is now used in the Official Food and Drug Administration sterility test. Taka-diastrase also possesses the inactivating property. Apparently, the demonstrated power of clarase and taka-diastrase to inactivate penicillin in sterility tests is due to bacterial end products which these preparations contain (95). A number of investigators (65, 97, 140) have recommended the use of standardized penicillinase preparations attained directly from bacteria in the sterility test. Some of these agents appear to be worthy of consideration. Still another substance that may be of value is cysteine (18, 71). This agent is also destructive to certain other antibiotics.

ANTIBIOTICS DERIVED FROM THE GENUS *Aspergillus*

The successful isolation of penicillin from different strains of *P. notatum* resulted almost immediately in the examination of many

other fungi for the presence of antibiotics. One of the earliest of these investigations (159) led to the isolation of aspergillic acid from a strain of *Aspergillus flavus*. This substance has been obtained in crystalline form and appears empirically to be $C_{12}H_{20}N_2O_2$. It has been reported to be effective against Gram-negative as well as Gram-positive bacteria. However, it is quite highly toxic to animals. When administered with mucin it has given some protection against infections induced by gonococci and may have value as a local application in gas gangrene. Another product of *A. flavus* is flavicin (16, 104, 145). Crude extracts of this substance are toxic. It is highly active against many Gram-positive bacteria and has many properties in common with penicillin. When obtained in a purified state so its chemical composition can be ascertained, flavicin may be found to be identical to penicillin. Still another antibiotic of *A. flavus* has been designated as "flavicipin" (106). This product was obtained from growth in submerged culture in a modified Czapek-Dox medium and has marked similarity to penicillin. Both are highly soluble, readily absorbed by living tissue and rapidly excreted by the animal body. Both appear to be equally susceptible to the enzyme penicillinase. When the chemistry of flavicipin is known, it may prove to be penicillin.

Investigations of *Aspergillus fumigatus* have led to the discovery of several antibiotics. One of the first to be obtained in crystalline form was fumigatin (5). It is quinoid in character, being 3-hydroxy-4-methoxy-2,5 toluquinone. Thus it is similar to another antibiotic, spinulosin (144), which is obtainable from this mold as well as from *Penicillium spinulosum*. Both are oxidizing agents and may be biologically active as a result of their ability to oxidize sulfhydryl compounds. Fumigatin is fairly soluble and quite active against Gram-positive bacteria. It is too toxic, however, to be of therapeutic value. *A. fumigatus* also produces gliotoxin, an agent first obtained from *Trichoderma lignorum* (155). It is a sulfur-bearing ring compound appearing as elongated plates in crystalline form. Its empirical formula is given as $C_{13}H_{14}N_2O_4S_2$ (84). Gliotoxin is described as being both bactericidal and fungicidal. It is exceedingly toxic (125) and possesses no antibiotic activity when tested *in vivo*. It is inactivated by cysteine, possibly by reduction of the disulfide group (99). Two other antibiotic substances isolated from *A. fumigatus* are fumigacin (150) and helvolic acid (22). These appear now (144) to be identical products.

Fumigacin has been obtained in the form of fine white crystalline needles. It is a heat-stable, monobasic acid bearing the empirical formula, $C_{32}H_{44}O_8$ (148). It is not particularly toxic for animals following single intravenous injections, but continued administration results in serious liver damage. This alone is sufficient to disqualify it as a valuable therapeutic agent.

Another product of the *Aspergilli* is clavacin (21, 150), obtained from *A. clavatus* and identical to claviformin from *Penicillium claviforme* (9), clavatin (160) and patulin from *P. patulum* (117). This substance attracted much interest when it was reported to have the power of curing colds (117). Thorough clinical trial, however, failed to verify these early observations (134). It is now known that clavacin is quite toxic (99), being a general protoplasmic poison. It is fungistatic and is effective against certain plant pathogens. The antibiotic activity of clavacin appears to be due to its reaction with sulfhydryl groups found in bacterial enzymes or in metabolites essential to bacterial metabolism. The antibiotic bears the formula $C_7H_6O_4$.

Still other members of the genus *Aspergillus* capable of elaborating antibiotics are: *A. giganteus*, which produces gigantic acid (116), an agent closely resembling penicillin; *A. parasiticus* producing parasitacin (29), and *A. candidus* elaborating citrinin (139). The last was first isolated from *Penicillium citrinum* (7, 70) and has been obtained as a yellow substance in crystalline form. The chemical formula suggested for it (32) is $C_{13}H_{14}O_5$. It is most highly active against Gram-positive bacteria, but like numerous other antibiotics is too toxic for animals to be of therapeutic value (125).

Although penicillic acid was first found as a product of members of the genus *Penicillium* (113), it is also produced by *Aspergillus ochraceus* (86). It is not to be confused with penicillin from which it differs markedly. The empirical formula for penicillic acid (112-114) has been suggested as $CH_3C(:CH_2)COC(OCH_3):CHCOOH$. It is quite active against the colon-typhoid group as well as against the Gram-positive bacteria.

Penicillin itself is not an exclusive property of the genus *Penicillium*. It has also been isolated from *Aspergillus niger*, *A. nidulans*, *A. oryzae* and *A. flavipes* (49).

Mutations of the *aspergilli* have been produced through chemical

induction (135) and by ultraviolet light (120). Many interesting variants have been obtained. Whether any of these has increased powers for elaboration of antibiotics is still to be determined.

AN ANTIBIOTIC SUBSTANCE PRODUCED BY *Chaetomium cochliodes*

Waksman and Bugie (146) have shown that a certain strain of *Chaetomium cochliodes* produces an antibiotic substance, designated as "chaetomin", that is highly effective against Gram-positive bacteria. The antibiotic principle is found both in culture filtrates and in the mycelium of the organism. It occurs in larger quantities in the mycelium than in the culture filtrate (58). Chaetomin is extracted from culture filtrates by ethyl acetate and from the mycelium by acetone. It is repurified by washing with sodium bicarbonate and sodium carbonate, and is further purified by treatment with petroleum ether and by chromatographic absorption. It contains nitrogen and sulfur and, in some respects, is similar to tyrothricin, an antibiotic of bacterial origin.

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PLANT DISEASES DURING THE YEARS 1941-1945 IN THE UNITED STATES AND CANADA

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The achievements of American agriculture during the war years have been widely acclaimed. The part played by the abundant—often record breaking—crops produced on this continent in supplying food to our fighting forces and to allied civilian populations needs no further comment. The fact is too little known, however, that during this period there were few disease outbreaks which caused critical losses in major food crops in the United States or Canada.

No reader of this journal will be tempted to misconstrue the foregoing statement. Plant diseases certainly reduced the yield of many, probably of most, crops. The losses were, however, usually not dramatic. There was nothing approximating the epidemic of stem rust of wheat which in 1916 cut American wheat yields more than a third and tragically upset national and world food plans. The major losses sustained from plant diseases were of a quite different sort. They were of the type which students of plant diseases often seriously discuss but too rarely mention in print. For example, in the opinion of some of the keenest and most experienced plant pathologists the yields of important clonal crops are regularly cut from 50% to 60% by root rots. However, not even the editor of *The Botanical Review* is likely to call for a summary of unpublished estimates of losses from plant diseases. This article, like its two predecessors (12, 13), must be based largely on information already available through publication. Thus the choice of the diseases to be included must be determined largely by the availability of records. Numerous colleagues in the profession have been consulted and have made helpful suggestions as to which diseases should be mentioned.

CANADA

To summarize, in the usual sense of the word, so concise and inclusive a series of reports as those of the Canadian Plant Disease Survey is difficult (1-5). Records of a few diseases have been

selected from the wealth of material available in the hope that they represent those of most widespread importance and interest.

Cereal Rusts

The situation with respect to losses from cereal rusts in Canada is largely a mirror of the use of resistant varieties. Indications of potential severity were largely obtained from experimental plots and from scattered plantings of the older non-resistant kinds. Through 1940, 1941 and 1942 stem rust of wheat caused little damage in western Canada and only local damage elsewhere. In 1943 it was rather more severe in eastern Canada, as was stem rust of oats. The situation in 1945 underscores the whole problem of cereal rusts, for experimental plots indicated that in Manitoba at least a severe epidemic of wheat stem rust would have resulted save for the existence of varietal resistance. Likewise, the severity of oat stem and crown rust damage on hitherto resistant varieties emphasizes the ease with which new races of parasites develop. Leaf rust of wheat, after several years of small importance, showed a mild increase in 1944, and carried on in 1945. Here, too, formerly resistant varieties were affected, probably indicative of attack by new races of the rust.

Cereal Root Rots

The most complete reports of damage from common root rots on wheat and other cereals, caused by *Helminthosporium sativum* and *Fusarium* spp., are those of Manitoba, Alberta and Saskatchewan. Manitoba, with an estimated reduction in yield of 7.4% in 1939, showed 16.6% in 1940 and 12.1% in 1941 (2: iii). Comparable percentage figures for subsequent years are not given, but there seems to have been a downward swing through 1942 and 1943, followed by much more severe damage in 1944. In Alberta fluctuations during this period were less well defined, though the disease was by no means absent from this area. Calculations of a "disease rating" in Saskatchewan show that while damage in 1941 and 1943 was about the same, it was far less during the intervening year, a year of excellent crop production. In 1944 records indicate somewhat less loss than in 1943.

Browning root rot (*Pythium* spp.) in Saskatchewan was of particular significance in 1942, although recovery from early lesions

was general. Certain areas of all three provinces were severely affected in 1943.

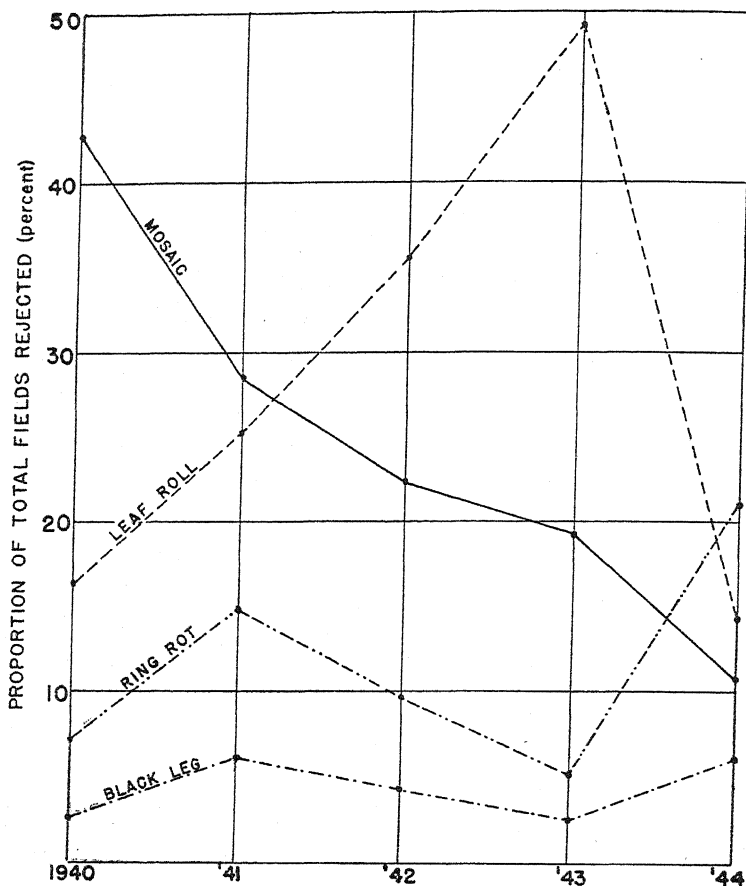


FIG. 1. Fields rejected by the Canadian seed potato inspection service, 1940-1944. Lines represent percentages of total fields rejected because of the four diseases indicated.

Potato Diseases

The seed potato inspection service makes possible a fairly accurate survey of four potato diseases in Canada: mosaic, leaf roll, blackleg and ring rot. Further condensation of the available data appears in Fig. 1, in which the percentages represent the proportion of total fields rejected which resulted from the occurrence of the particular

disease indicated. A graph prepared to show the percentage rejected out of the total number of fields inspected would result in much lower figures. Total rejections for all causes during the five years, 1940-1944, are 30.0%, 34.7%, 36.8%, 42.3% and 11.0%, respectively. Exact data are not yet available for 1945, but ring rot is mentioned as "still one of the most important diseases of potato", and a local severe epidemic of leaf roll in New Brunswick is recorded.

During the period of this survey, late blight has been of considerable importance. In Manitoba, after a period of relative quiescence, it was considered of epidemic proportions from 1941 through 1944; and no year during that period failed to show one or more rather severe outbreaks in other parts of the country. Preliminary reports indicate that the disease was less important in 1945.

Fruit Diseases

Fire blight of apple and pear has occurred somewhat irregularly during the past five years. In 1941, for example, it appeared significantly in Ontario and southwest Quebec; in 1943 in Alberta, Manitoba, Ontario and Quebec; and in 1944 in restricted areas of Alberta, Saskatchewan and Manitoba. It is a disease largely held in check by careful horticulture; and one capable of severe inroads if neglected.

Like fire blight, scab has been of irregular occurrence. Some significant outbreaks were noted as follows: 1941, New Brunswick, lower peninsular and Vancouver Island, British Columbia, and Prince Edward Island; 1942, southwest Ontario; 1943, all of eastern Canada; and 1945, from Ontario eastward. More than once an unavoidable break in the rigid spray schedules seemed to account for the subsequent difficulties.

Both X-disease of peach and its related but possibly somewhat variant western X are now present in Canada. The former was first recorded on the Niagara peninsula in 1941, where it had apparently been present for several years; and a suspected occurrence of western X in the Okanagan Valley was confirmed in the same year. Spread in both localities has been limited and slow, particularly with respect to X-disease in the Niagara area. By 1944 a maximum of 7.7% infection was attained by western X virus among 2,457 trees of 10 mapped orchards.

Some records of new or significant occurrences in Canada during these years may well be noted:

- 1941—A case of potato wart (*Synchytrium endobioticum*) was found in a small garden in Halifax (2: iv).
- 1942—Wisconsin leaf spot of tobacco (*Pseudomonas mellea*), new to Canada, was epidemic in the southern tobacco-growing area of Quebec (3: v).
- 1943—Tobacco downy mildew (*Peronospora tabacina*) was reported in seed beds of two areas in Essex County, Ontario. Previous records exist for Kent County in 1938 and 1940 (4: 72, 1: 54).
- 1944—The Dutch elm disease (*Ceratostomella ulmi*) was discovered near Sorel, Quebec, late in 1944. Before the close of the season 28 diseased trees were located in an area about 40 miles long near Lake St. Peter, about 50 miles below Montreal (5: 102).
- 1945—Crown wart of alfalfa (*Urophlyctis alfalfae*), new to Canada, was recorded at the University of British Columbia and at Chilliwick (Unpublished manuscript).

UNITED STATES

Late Blight of Potato

Just a century before the period now under review, late blight of potato, caused by *Phytophthora infestans*, was the most discussed plant disease in North America (10). It was first reported here in 1843, and during that and the two subsequent years caused serious, even terrifying, losses in the northeastern states. As is well known the disease was an important cause of the Irish famine, then at its height. Late blight has been recognized as of major importance both here and in Europe ever since and is the subject of a voluminous literature. It was one of the first plant diseases to be successfully controlled by the then newly discovered Bordeaux mixture (1882-1885). Yet in the years just past it has caused serious losses in an important food crop and has appeared in parts of the United States from which it had not hitherto been recorded.

As a result of this and the general situation described in the opening paragraphs, potato late blight was again the most discussed plant disease on this continent. Losses in the 1941 crop seem to have been relatively small. Of the upper Mississippi Valley

states only Wisconsin reported significant losses, 4%. Ramsey (26: 451) confirms this by reporting that on the Chicago market Wisconsin stock in October frequently averaged over 10%. He also found an average of 12% loss in many loads from Florida and 3% to 10% in several cars of California potatoes received in May. Of the eastern states only Pennsylvania (25: 388) reported serious losses from late blight. This was in the northern section of the State.

In 1942, however, losses from late blight were very heavy in many areas. On the map (Fig. 2) are shown the estimated losses

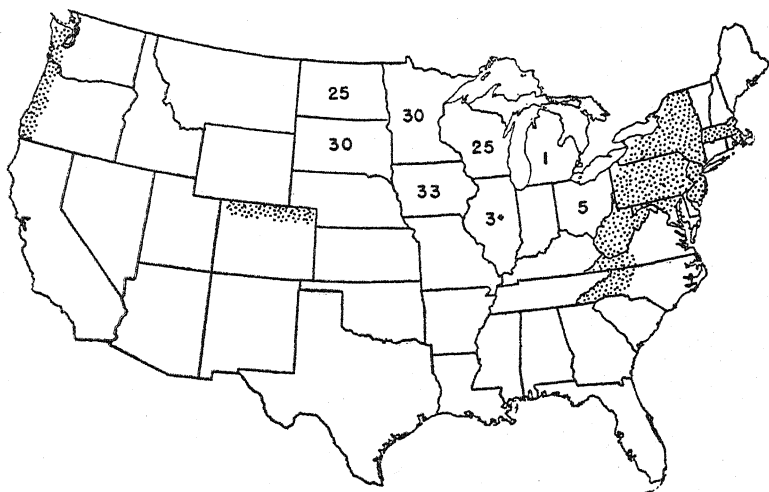


FIG. 2. Losses from potato late blight, 1942. Numerals represent estimated percentage losses for state indicated (Melhus); stippled portions indicate regions from which losses were reported simply as "serious".

for the various states in the upper Mississippi Valley, as compiled by Melhus (9). Shaded portions indicate regions from which losses were reported as "serious", with no estimate. For Garrett County, Maryland, the loss from this disease was estimated at 40% to 50% of the crop (26: 383). Weiss (27: 203-206) prepared a summary of the potato situation that year, based largely on the statistics of the Crop Reporting Board for the 12 largest potato-producing states, which normally grow about 80% of the total United States crop. The eastern states in this group showed a decrease of five and one-half million bushels as compared to 1941, the crop of which year was itself below average. Late blight was, ac-

According to Weiss, "almost certainly the principal factor in this curtailment of production", since weather conditions were generally favorable for high potato yields. However, the "superlative production" of the far western states, which showed an increase in the late or main crop of more than 12 million bushels, as compared to 1941, correlated with exceptionally favorable weather, kept the national situation from being much more serious than it was. That late blight of potatoes is one of those diseases which flourishes with the same temperature, moisture and other conditions as are favor-

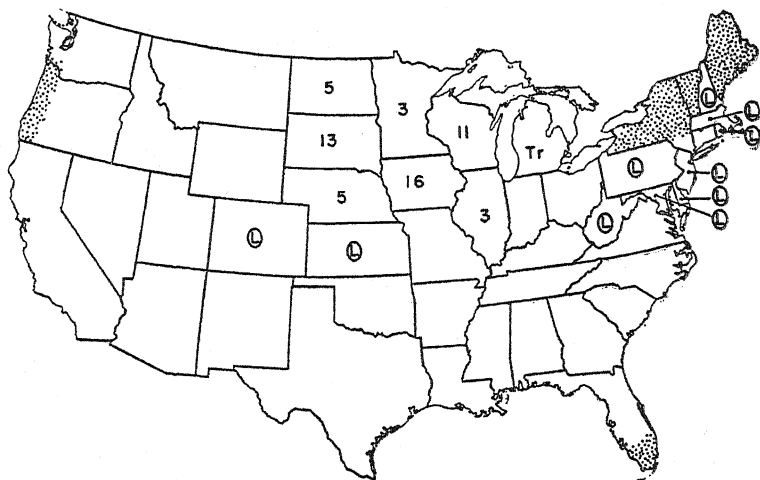


FIG. 3. Losses from potato late blight, 1943. Numerals represent estimated percentage losses for state indicated; "Tr" indicates "trace" (Melhus). Stippled portions indicate regions from which losses were reported as "considerable" or "worse than usual"; the letter "L" states in which reported losses were relatively light.

able to the host itself, has long been known and was discussed in 1937 (7).

Specific mention may be made of the fact that in 1942 (27: 18, 19) late blight occurred in Colorado in serious amounts for the first time on record. An estimate of 20% loss due to tuber rot was reported for certain irrigated sections of northern Colorado. Relatively small amounts occurred in the same area in 1941, the first time the disease was found in Colorado. In 1942, also, the disease was reported for the first time for Nebraska (26: 395) and North Dakota (26: 372).

Losses from late blight in 1943 in the upper Mississippi Valley were, as shown by the map (Fig. 3), generally much lower than in the previous year. Many other states reported light losses. In Vermont, however, the disease was said to be "worse throughout the state than in many years", with an estimated loss in storage from this cause of 13% (28: 1063). Storage losses from the late blight were very heavy in Maine also. A survey by R. C. Cassell showed that approximately 10% of the potatoes stored in Aroostook County

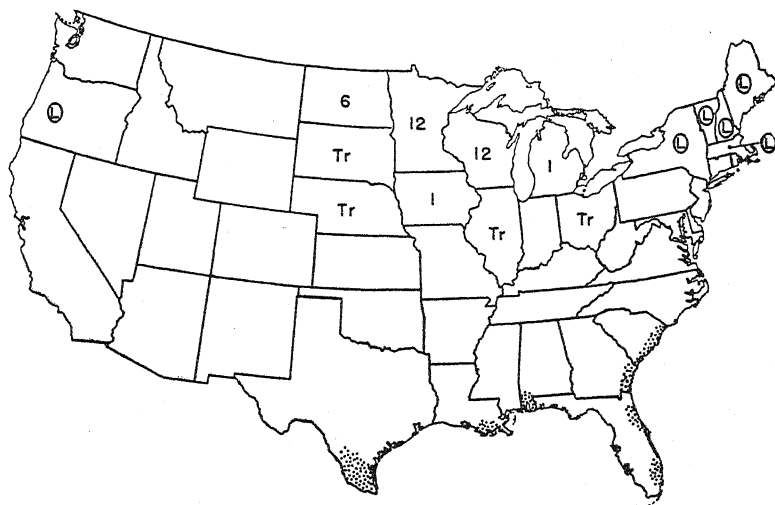


FIG. 4. Losses from potato late blight, 1944. Numerals represent estimated percentage losses for state indicated; "Tr" indicates "trace" (Melhus). Stippled portions indicate regions reporting very heavy losses; the letter "L" states in which reported losses were relatively light.

rotted in storage because of late blight (28: 293-297). The Maine crop for 1943 was 73,485,000 bushels. The disease was "very severe in Oregon". Losses from late blight, estimated at 25% to 33% of the crop, occurred in the Belle Glade, Florida, fall crop in 1943 (28: 41). Hitherto, late blight has occurred only in the spring crop, that is, in March and April.

For the season 1944, outside the area covered by Melhus' review, the disease was reported as rather light in most northern states (Fig. 4). In some southern states, however, losses were very heavy. The region around Charleston, South Carolina, reported prospects for harvesting 10% of a normal crop (28: 438). Around

Savannah, Georgia (28: 522), 75% to 100% of the potato plants were dead from late blight. Eddings (28: 439) reported that in the important potato area around Hastings, Florida, a conservative estimate of loss was 20% of the crop, the greatest in 13 years. It was also serious in southern Florida (28: 361) and in the lower Rio Grande Valley of Texas (28: 247). The severity of the loss in Louisiana may be judged from the estimate made by A. N. Moreau, County Agent, that over 800 acres of potatoes had been destroyed in La Fourche parish. Bain adds (28: 433) that at the same place a few cars of potatoes on the tracks had been re-loaded one or more times because of rotting.

The history of potato late blight in Baldwin County, Alabama, on the Gulf Coast, east of Mobile Bay, has been recorded (29: 378). The first authentic record of its occurrence was a trace in 1940. Following this there was a gradual increase in its prevalence until 1944 when more than 60% of the crop was destroyed.

For the year 1945 potato late blight was reported as severe in Massachusetts: "Worst I have even seen" (29: 654). Specific estimates of losses are for Long Island, New York, "30 per cent of the fields down", and Kansas, 10% loss. More noteworthy, perhaps, are the reports of this disease in unusual localities. In June, T. W. Bretz reported that from 30% to 50% of the foliage had been killed in fields in Clay County, Missouri; this is said to be the first known occurrence of late blight in that state (29: 549). The same month Valleau reported it for the first time on the early crop of potatoes in Kentucky.

In the southeastern states, generally, the disease was present and often severe. Growers in South Carolina estimated that it would destroy 30% of their crop (29: 44), and in southern Alabama the estimated loss was 30% to 40% of the late crop, but very little in the early crop. The disease was also reported from southern Mississippi and southern Louisiana (29: 444). Losses from this disease were heavy in the area around Brownsville, Texas (29: 234).

Stem Nematode of Potato

A hitherto unknown disease of potato caused by a nematode was discovered in Idaho in 1943. The first report of the disease was made by Earle C. Blodgett (27: 658, 659) who gives credit to various associates for their part in the discovery. The causal nema-

tode was tentatively identified as *Ditylenchus dipsaci* by G. Steiner. A later report (29: 58) suggests that it is an undescribed species. The most conspicuous symptom is a "skin checking" which is followed by decay. Although found in only two fields, injury estimated at 50% to 75% was reported. In October 5-16, 1944, a survey of potato diseases with particular reference to the stem nematode was made near Aberdeen, Idaho. This parasite was identified in six fields, three adjacent fields in one group, two adjacent in another, and a new location with apparently no connection with the others (29: 58-60).

Yellow-spot of Wheat

In the course of a survey made in June, 1941, in central and western New York, M. F. Barrus found a leaf spot of wheat (26: 246) not hitherto reported for the United States. This disease, which was also observed by Barrus in 1940, is caused by a species of *Helminthosporium* identified by A. G. Johnson as *H. tritici-vulgaris* Nisikado, first described from Japan. Barrus reported the occurrence of the disease on 35 fields scattered throughout eight counties and observed that the loss in yields caused by it was probably small.

In the same month A. G. Johnson found this disease in commercial fields of winter wheat in Montgomery and Howard counties, Maryland. He also found it in the winter wheat breeding nursery at Beltsville, Maryland, where it attacked many hybrids and strains that are resistant to leaf rust—causing more injury than leaf rust causes on varieties susceptible to it. Johnson adds that the "Indications are that the disease has been with us for some time and that it has been overlooked because of its resemblance to the leaf spot caused by *Helminthosporium sativum* Pamm., King, and Bakke. This disease was subsequently found in Virginia, West Virginia (28: 209), Kansas (29: 529) and Nebraska.

Stem Rust of Wheat

Our best information regarding losses from stem rust of wheat (*Puccinia graminis tritici*) comes from the Office of the Conference for the Prevention of Grain Rust, D. G. Fletcher, Secretary. Its loss estimates for the 13 barberry eradication states (Colorado, Indiana, Illinois, Iowa, Michigan, Minnesota, Montana, Nebraska, North Dakota, Ohio, South Dakota, Wisconsin, and Wyoming)

expressed in bushels are 1941—1,200,000; 1942—Trace; 1943—934,000; 1944—16,117,000; 1945—1,027,000. Nineteen million bushels is, of course, a lot of wheat, especially to anyone writing in May, 1946. When, however, it is realized that these same states produced 572 million bushels in 1945, it becomes evident that the percentage loss from this disease was fortunately small in that area during the period now being considered. In Virginia, however,

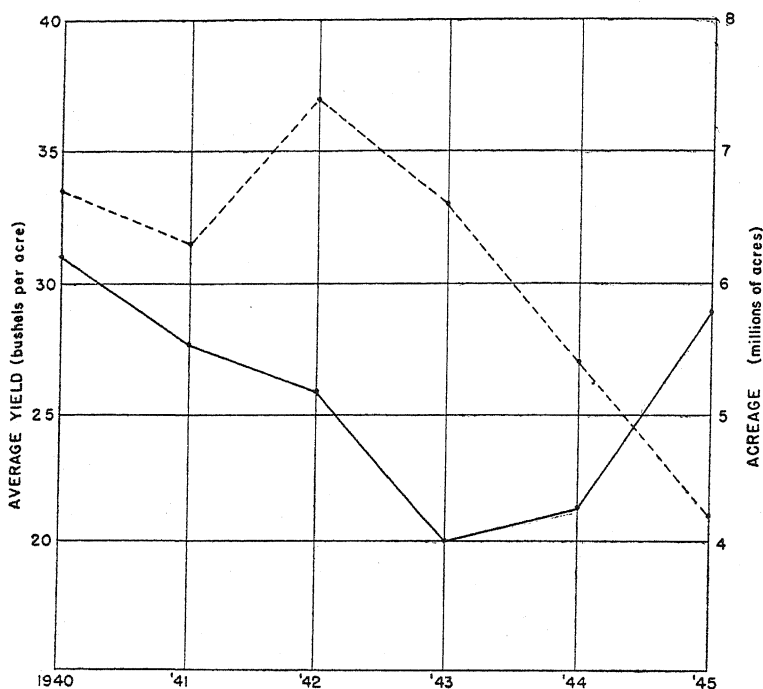


FIG. 5. Barley production, 1940-1945. Solid line indicates average yield per acre in seven midwest states, plotted against vertical scale to left. Broken line represents total plantings of barley in these states for the same period, plotted against vertical scale on right.

S. B. Fenne reported that in 1942 stem rust of wheat had been more severe than at any time for the last ten years. A conservative estimate placed the loss for the state at 10% (26: 479).

Barley Yields

The sharp decline in barley production in the last few years has been a matter of major concern to those commercially interested. The Midwest Barley Improvement Association—John H. Parker,

Director—has furnished the figures on production in seven mid-western states: Illinois, Wisconsin, Michigan, Iowa, Minnesota, North Dakota and South Dakota (Fig. 5). The serious decline in both acreage and production has occurred in the five states first named above. The Dakotas have largely maintained both acreage and production. In the opinion of many of those interested, this decline is largely due to root rots combined with unfavorable weather.

Spread of Cucurbit Downy Mildew on the Atlantic Coast

During recent years increased attention has been given to the relation between the abundance of a disease-producing organism in the southern states and its time of appearance, abundance and destructiveness further north. Stem rust of wheat and tobacco downy mildew are cases in point. So also apparently is the cucurbit downy mildew (*Peronosplasmopara cubensis* B. and C. Clint). This fungus is known to live through the winter in Florida. Several investigators (some of which are cited by Doran (6)) have suggested that spores from this obvious source may well be disseminated northward, possibly by successive stages, as the season advances and crops are planted farther north. Doran states that (6: 8):

"In each of the seven years from 1924 to 1930 inclusive, downy mildew of cucumber has been found in the field in Massachusetts, and has always appeared within the first 18 days of August, whether the summer was rainy or dry. But the disease was not found in this state in 1931 although many cucumber fields were examined at weekly intervals, or oftener, up to the time of killing frosts. The weather of the season [in Massachusetts] was not significantly different from that of previous seasons in which downy mildew had been severe, and the absence of the disease in 1931 is attributable only to lack of primary infection rather than to failure of the disease to spread within a field".

On the basis of correspondence with experienced observers in other states, he published the further information that the disease was not found until the very end of the season and then in but one field in Connecticut. It was not reported in New York or Pennsylvania. There was very little in Delaware, in South Carolina and in Georgia. Doran considers particularly significant the fact that in Florida the fields were remarkably free from downy mildew and the disease not of economic importance.

For some years prior to 1941, Charles J. Nusbaum at Blackville, South Carolina, had made predictions of the time of appearance of cucurbit downy mildew in that region. These predictions were

based on reports from M. N. Walker at Leesburg, Florida, and A. N. Brooks at Lakeland, Florida. In 1941 a somewhat more formal reporting service was organized including pathologists in each of the Atlantic coastal states from Florida to Massachusetts. These reports for the past three years have been summarized in the Plant Disease Reporter (28: 82-29; 141), and are presented in tabular form below.

The year 1944 holds the greatest interest. The disease was destructive in Florida and near Charleston, S. C. (29: 143). It appeared elsewhere in the Carolinas and Virginia but was checked by hot dry weather. States north of Virginia were virtually mildew-free in spite of the fact that according to Boyd the weather in Massachusetts was favorable for such fungi. He cites specifically the appearance of potato late blight in early August. A reasonable explanation of the failure of downy mildew cucurbit to appear in the northern states is that no inoculum was present.

DATES ON WHICH DOWNY MILDEW OF CUCURBITS WAS FIRST NOTED
IN THE LOCALITY INDICATED

State	1943	1944	1945
Florida	Present about the usual time	Developed on fall cucurbits; observed throughout the winter	April 20
South Carolina (Charleston)	June 1	May 31	May 9
South Carolina (Blackville)	June 15	June 10	May 25
North Carolina	June 30	June 27	June 8
Virginia	July 26	August 1-7
Delaware	July 19	Not noted	July 26
Pennsylvania	Not noted	August 8 (late July)
New York (Long Island)	1 field, probably infected in late July
Connecticut	August 1
Massachusetts	August 25	None found in spite of favorable weather conditions	July 26

Downy Mildew of Tobacco

Tobacco growers and plant pathologists alike have become so accustomed to downy mildew (*Peronospora tabacina*) that reports

State	REPORTED LOSSES FROM DOWNY MILDEW ON CUCURBITS		
	1943	1944	1945
Florida	Losses light.	Losses moderate to severe.	Losses light.
So. Carolina (Charleston)	Late cucumbers damaged considerably. Plants dead by June 21.	Cucumber fields severely defoliated.	50% defoliation serious damage.
So. Carolina (Blackville)	Cantaloup yields reduced 25%—Cucumbers escaped.	Cucumbers escaped. Late cantaloups reached a destructive level.	Cucumbers escaped—Cantaloups suffered considerably—complete defoliation by July 1.
No. Carolina	Cucumbers escaped. Cantaloup suffered late in season.	Cucumbers escaped. Late cantaloups suffered.	Damages light in early fields—moderate to heavy in late plantings.
Virginia	Only late cantaloups and watermelons suffered.	Very little damage, few watermelons affected.
Delaware	Losses very light.	No infection.	Cucumbers escaped—cantaloup damage not reported.
Pennsylvania	No infection.
New York (Long Island)	No loss.
Connecticut	Destructive on some cucurbit crops.
Massachusetts	Late cucumbers 25-90% loss. Late summer squash from 0 to 25% loss.	No infection (possibly no inoculum).	Losses moderate.

of losses from the disease are regrettably infrequent. The available information does indicate that the disease was relatively quiescent in 1941 and 1942, very active in certain localities in 1943 and 1945, intermediate in 1944. Virginia (26: 52), North Carolina (26: 111) and South Carolina (25: 203) indicate slight losses in 1941. The Massachusetts report for 1941 is "somewhat more severe than in past seasons" (26: 7).

The 1942 report for North Carolina is specific, "less severe in 1942 than in any previous year since its occurrence in the flue cured belt of North Carolina" (27: 493). The disease was again active in Massachusetts (27: 145) but generally light in Pennsylvania (26: 432), Maryland (26: 243), Virginia (26: 432), Georgia (26: 121) and Florida (26: 223).

In 1943 tobacco downy mildew was more severe than in any of the past three seasons in both South Carolina (27: 208) and Georgia (27: 174). The Georgia report adds "Georgia is experiencing the most critical shortage of tobacco plants since 1932". The cause was the same, a combination of downy mildew and freezes. The report from Virginia was "losses severe and unusual, felt not only in plant beds but throughout the growing season". Losses in that part of the crop planted after May 25 were estimated at 20% to 50% (27: 643).

Only in Virginia and the Carolinas was severe damage reported in 1944. In 1945, however, the disease reached southern Ohio for the second time on record. The only previous outbreak was in 1938 (29: 558). In the southwestern counties along the Ohio River losses were very heavy. In the cigar area 50% to 80% of the plants were killed. In Kentucky the disease was general and destructive in tobacco beds throughout the state (29: 719), although none had been seen in Kentucky in 1944. A negative report of great interest to those who have followed the history of this disease in the United States is that of Bain, that none was found during 1945 in the tobacco area at Grande Pointe (St. John's Parish) (29: 20; 29: 299). It will be remembered that downy mildew appeared in Louisiana in 1931 and has not been found there since.

Some indication of the too little noticed cost to American agriculture of such a disease as this is found in the reports (26: 111; 29: 270, 390) that "Most growers now plant double the area

of the bed space that they planted before the appearance of downy mildew". Significant also is the fact that delays in planting often cause severe losses. For example, in 1944 a two weeks' delay in planting in the "border belt" of North and South Carolina was estimated to have cost many growers \$175 an acre.

Asparagus Rust

The rust-resistant Washington strains of asparagus were introduced by Norton in 1919. They were so successful that for a number of years asparagus rust (*Puccinia asparagi*) was little more than a curiosity. The rust has, of course, persisted, and there is evidence that it is again becoming, at least locally, severe. According to Fulton (27: 19), asparagus rust has been slowly increasing in Illinois since 1930. It reached a peak of severity in the summers of 1940 and 1941. In 1940 large acreages in northern Illinois were completely brown by the first of August as a result of this disease. During the summer of 1941 the rust was again severe and young fields were badly damaged in mid-summer. Other fairly recent serious outbreaks are mentioned in this paper. A severe outbreak of asparagus rust was observed in California in 1925. Massachusetts reported 2% loss from this disease in 1934, 3% in 1935 and 4% in 1936, with heavy damage on Washington strains not uncommon.

Further indication that asparagus rust is now of real economic importance comes from a survey made in Delaware in November and December, 1944, by A. J. Mix (29: 28, 29). He reports that of 18 fields examined in the Camden-Wyoming area, six were lightly infected with rust, four moderately and eight severely. Rust was also reported as doing some damage to asparagus in nine fields in the Bridgville area. Tidd, who surveyed the Illinois asparagus growing area with M. B. Linn in September, 1944, reports (28: 1049, 1050) that the rust was again wide-spread and causing considerable damage.

Cranberry Rots in 1942

No minor crop was watched with closer interest in the United States during the war than the cranberry. The announced determination of the government to give everybody in uniform a "genuine"

Thanksgiving and Christmas dinner placed this American fruit in a special class. It may then be worth mentioning that the Massachusetts cranberry crop in 1942 showed a high percentage of fruit rot. In this respect it was one of the four poorest crops of which we have a record. Competent observers characterized it as "the worst since 1933".

In his paper summarizing over 20 years study of the keeping

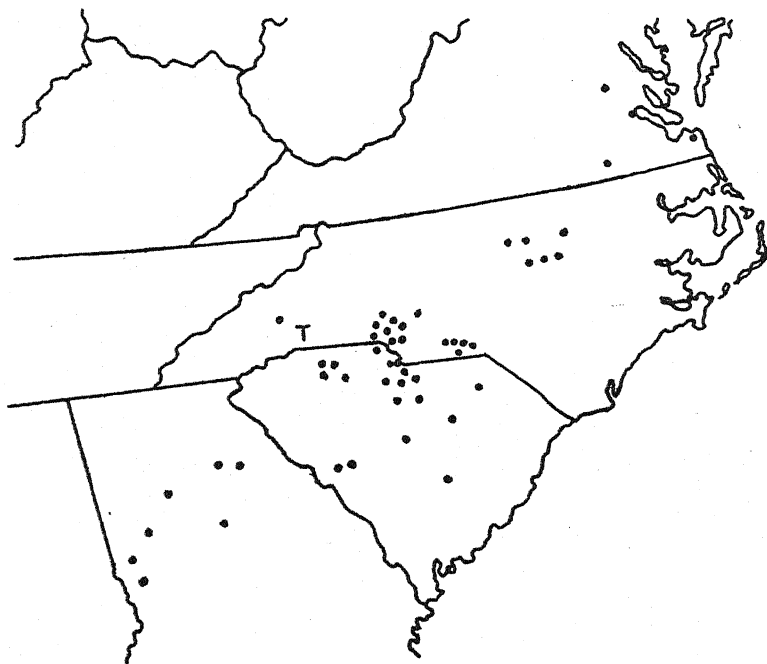


FIG. 6. Areas known to be infested with mimosa wilt, 1940 (Toole). Each dot represents one or more infected trees; the letter "T" indicates Tryon, N. C., where the disease was first established.

quality of this crop, Stevens said (11: 75) "... the worst possible combination of circumstances from the standpoint of keeping quality would be high temperature in May and June, a greater than normal number of days having 0.01 inches of rain in both July and August and an unusually large crop". This combination of factors has occurred four times in the last 25 years—in 1914, 1931, 1933 and 1942.

Wilt of "Mimosa" Tree

A vascular wilt of the so-called Mimosa tree (*Albizzia julibrissin* Duraz.) was reported in 1936 (8). This tree, a native of eastern Asia, is widely planted in the southern states as an ornamental. At

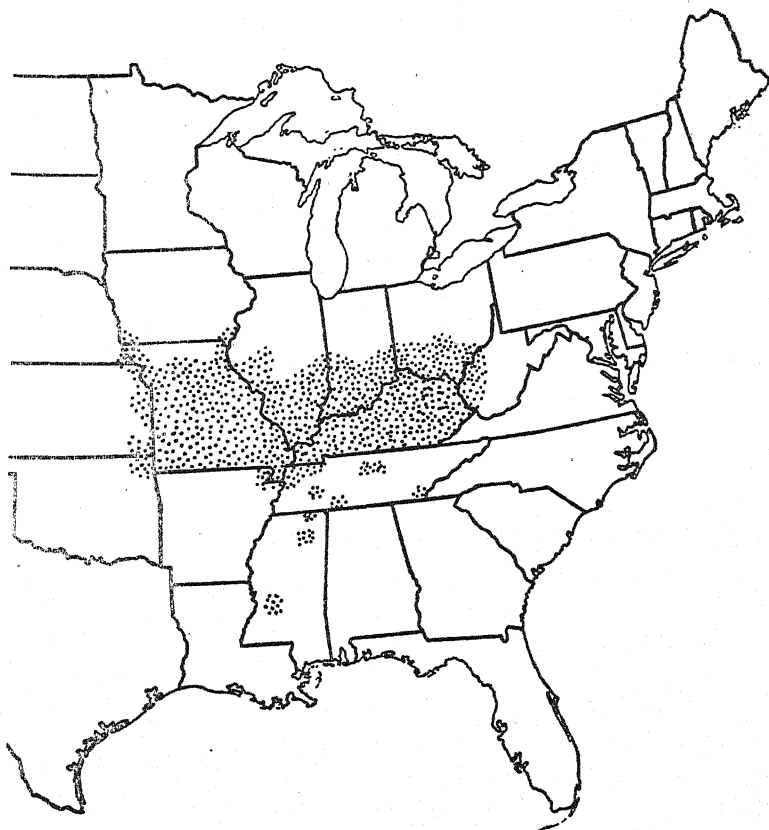


FIG. 7. Known distribution of phloem necrosis of American elm, 1945 (Bretz and Swingle).

the time of Hepting's study, several hundred trees were dead or dying in Tryon, North Carolina, where the disease may have been present since 1930. Additional infections were found in Virginia, North Carolina, South Carolina and Georgia. A more complete survey was made by E. Richard Toole in 1940. His map (25: 317), reproduced herewith (Fig. 6), gives the known distribution of the disease at the end of that season.

The first external symptom of the disease is the wilting of the leaves on some of the branches. By the time wilting takes place, a brown ring or partial ring of discolored sapwood can be found in the trunk. Hepting (8) proved the disease to be due to a hitherto undescribed species of *Fusarium* which he named *F. perniciosum*.

A disease of this tree having identical symptoms was described from the southern Soviet Union in 1920. The severity of the disease is indicated by the fact that by the middle of August, 1938, the mortality of the street trees of Tryon had been about 70%. It was reported that hundreds of trees were dead or dying along roads in other localities. No evidence was found to indicate that trees outside the Tryon area were diseased before 1934.

Phloem Necrosis of Elm

The known range of phloem necrosis of elm in 1945 is shown on the accompanying map (Fig. 7). This disease was observed in Ohio in 1918. Twenty years later (1938) its infectious nature was proved by grafting experiments (14: 7). No vector has yet been discovered. As early as 1942 Swingle reported that 50% to 75% of the elms in some cities and towns had been killed by this disease.

The external symptoms of phloem necrosis are too general for accurate diagnosis of the disease. They are described as drooping and curling of leaves, gradual decline throughout the entire crown, or death of individual branches, followed in most cases by death of the tree. Such abnormalities might easily arise from drought, from girdling or from other diseases. Diagnostic symptoms are found in the discoloration of the inner bark. The phloem is at first yellow, later becoming a butterscotch or "raw-sienna" color. The moderately discolored phloem tissue has a faint odor of winter-green which cannot be detected in the bark of healthy American elms.

Students of the disease are inclined to believe that the disease may have been prevalent in parts of Indiana, Illinois and Kentucky for many years. A similar epidemic of dying elms was reported in Kentucky by H. Garman in 1893 and 1899, and in southern Illinois by Stephen A. Forbes in 1912. Some of the offspring of Kentucky elms which survived the epidemic of 1893-1899 appear to be highly resistant to phloem necrosis.

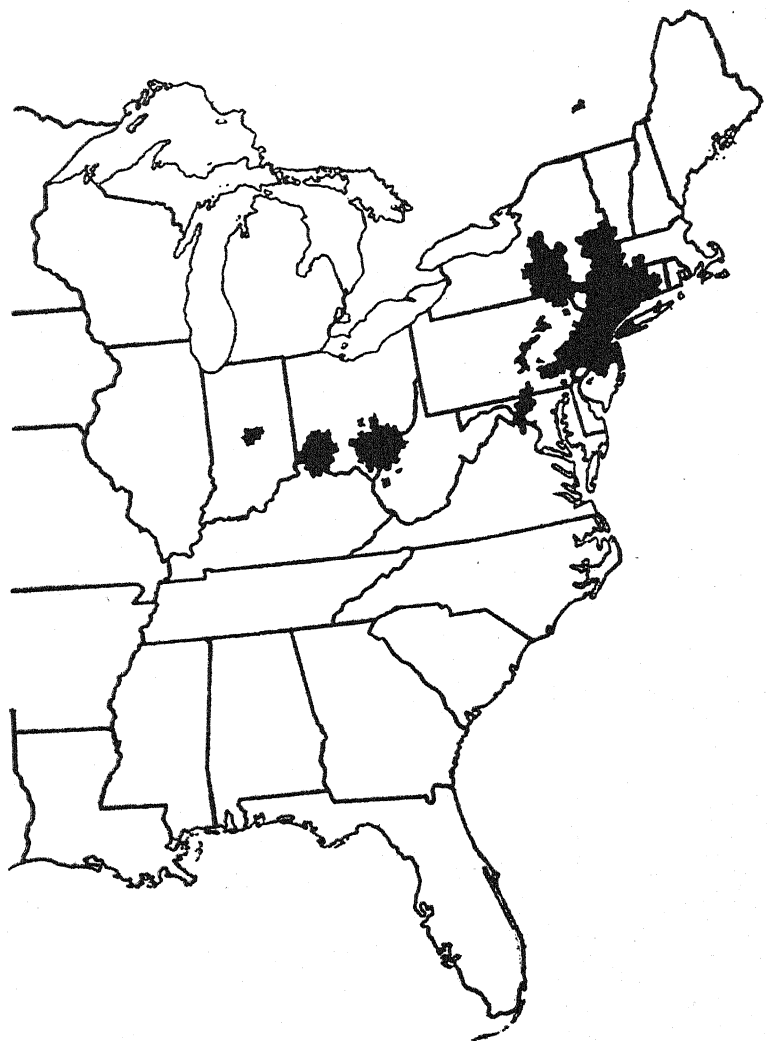


FIG. 8. Known distribution of Dutch elm disease, April 1946. Localities now "inactive" have been omitted.

Dutch Elm Disease

The accompanying map (Fig. 8) shows the known distribution of the dutch elm disease in April, 1946. This map was compiled from the monthly and annual reports of the Bureau of Entomology

and Plant Quarantine, Dutch Elm Disease Control Division. A comparison of this map with that published five years ago in *The Botanical Review* (12: 715) shows continued spread of the disease in the whole area centering in New York harbor. In central Maryland and especially in southern Ohio and the nearby regions of adjoining states the increase in the area in which the disease exists has been marked, even tragic.

Continued Recovery of Eel Grass

Eel grass (*Zostera marina* L.) was almost completely destroyed on both sides of the Atlantic in 1931-1932 by the so-called wasting disease. Absence of a disease or its decline are always less exciting and less likely to be recorded than its onset. However, the continued interest of two workers makes available records of the gradual return of this native plant in the coastal waters of North America. Cottam summarized reports from ten states and Canada in 1944 as follows (29: 302-310): "The situation along the United States coast is perhaps least favorable in the more open bays and estuaries of New Jersey and Maryland, and most favorable in the sandy loam areas of reduced salinity of Chesapeake Bay, Long Island, and part of the Maine coast. Though the situation in any local area is still highly variable and unpredictable, the trend is toward restoration of the plant in all favorable areas along the coast; consequently, we may confidently hope for an eventual complete return of the eelgrass". Dexter (29: 702-704) reports from one locality in Massachusetts and one in Connecticut. He notes "Thin windrows of washed-in eelgrass were observed in numerous places along the shores of the [Mystic] river". It may be added here that for the first time since 1933 considerable windrows of eelgrass were washed ashore in Buttermilk Bay, which is the extreme northern portion of Buzzards Bay, Massachusetts, in July 1945.

Late Blight of Tomato in 1946

To justify its title this review should exclude records of all phenomena occurring after 1945. The year 1950, however, seems relatively remote, and the temptation to include a 1946 report of unusual interest and completeness is too great to be resisted. The Plant Disease Survey of the U. S. Department of Agriculture has made available in Supplement 165 to the *Plant Disease Reporter*, much infor-

mation regarding the outbreak of late blight of tomato caused by *Phytophthora infestans*. Figure 9 which is reproduced from Fig. 1

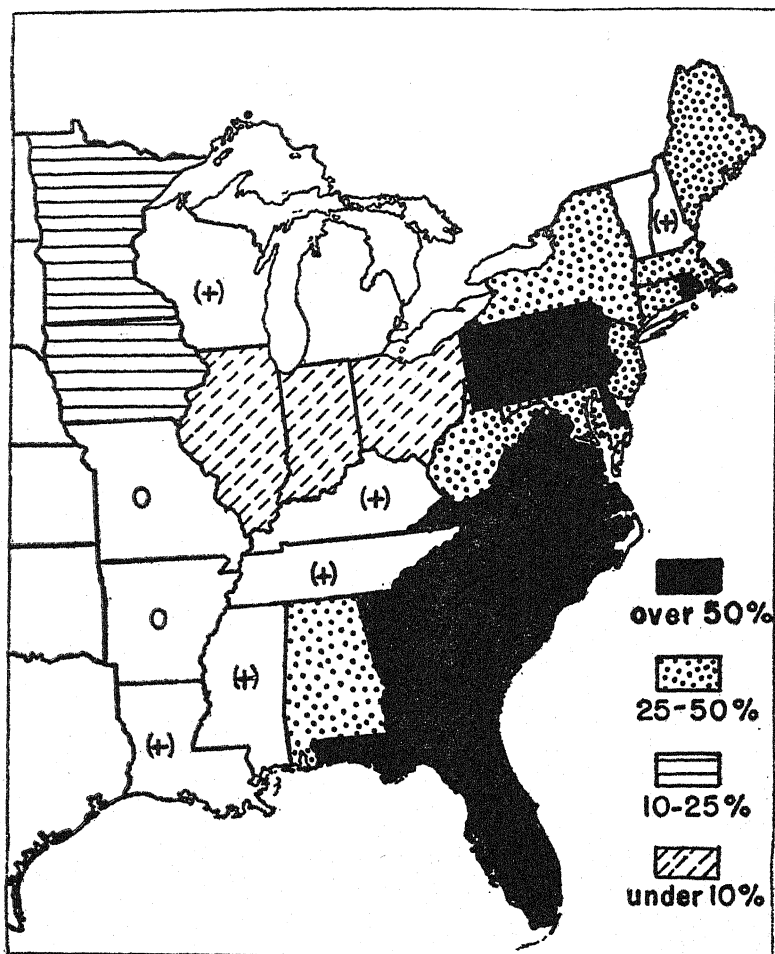


FIG. 9. Estimated losses from tomato late blight—1946. Extent of loss of individual states is indicated by shadings as shown in key to symbols. Plus signs indicate those states in which damage was appreciable, but for which no estimates are available. Zeros indicate states from which negative reports were received.

of the Supplement mentioned above gives the essential information.

Boyd, P. D. R. Supplement 165, 341–344, states incidentally, “Twice before in Mass. in 1905 and 1932–33, the late blight disease

of tomatoes assumed epidemic form and caused heavy losses in home gardens and commercial fields. In 1932, it even spread to and greatly damaged a great many fall greenhouse crops of tomatoes in eastern Massachusetts before the heating season started. This year, scarcely a garden or field in Massachusetts escaped damage. In 1932-33, the tomato late blight outbreak occurred in New England, with the most pronounced damage being in Connecticut, Massachusetts and Rhode Island. This year the outbreak in New England merely represented an aftermath or continuation of a similar situation that covered all the eastern states from Florida to New York state, and as far inland as Tennessee, Kentucky, Indiana and Illinois".

The most casual reader of this article cannot fail to notice that this unusual epidemic of late blight on tomato followed soon after several years in which late blight on potato was extremely widespread and destructive in the United States. It may be well, then, to restate here the opinion now generally held by plant pathologists regarding the relationship of the tomato and potato strains of this fungus. This is based on the work of Mills published in 1940. His conclusion was that the tomato strain arises in nature as a result of serial passage of the potato strain through tomato foliage. This conclusion was derived from the following observations and experiments. Tomato foliage is resistant to the potato strain of *P. infestans*, but susceptible to the tomato strain. A culture virulent for certain potato hybrids, gave the typical potato strain reaction on tomato foliage. After seven passages through tomato, it had acquired virulence for tomato equivalent to the tomato strain, while maintaining its original virulence for the potato hybrids. Further passages through tomato produced no observable further change.

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PLANT DISEASE CONTROL BY UNUSUAL METHODS

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This article, like the preceding (13), is admittedly incomplete. All that is attempted is a description of some of the less usual methods of plant disease control that have been suggested or have come into more general use during recent years. Naturally no claim is made that these methods are better merely because they are different. A new spray material or a new method of applying spray materials might actually have greater economic significance. If, however, the profession of plant pathology is to avoid stagnation, continuous search for new means of disease control must be maintained.

CONTROL OF POTATO LATE BLIGHT TUBER ROT BY KILLING THE TOPS OF THE PLANTS

Newly discovered methods of control are by no means restricted to plant diseases which are themselves newly recognized. Just a century after potato late blight was first discovered in this country, Bonde and Schultz (1) were working out a new method of control for the tuber rot it causes. As is well known, *Phytophthora infestans* often causes serious injury or death to the tops of potato vines. It is well known, also, that heavy losses from decay of potatoes in storage are frequently caused by the same fungus. In Aroostook County, Maine, 10% of the potatoes were destroyed in storage by this fungus in 1943. This loss to the nation has been placed as low as four million bushels. In view of the fact that the Maine crop that year was approximately 73.5 million bushels and that 85% of the acreage is in Aroostook County, the loss probably reached six million bushels.

Under certain conditions a major portion of infection of the tubers occurs at the time the potatoes are dug. This occurs chiefly, if not solely, if the tops of the plants (with, of course, some of the fungus still on them) are green when the potatoes are dug. This explains the facts long known in Maine and elsewhere that little or no rot occurs in storage when the potato plants are completely killed

by blight early in the season. On the other hand, the amount of rot in storage may be very large when there is only a little late blight in the field and the plants remain green until late in the season. The danger of digging the crop while there is still green foliage on the diseased plants has long been recognized. There are printed references to it at least 60 years old (1, p. 164).

Against this background of observed fact the newest means of reducing tuber rot has been developed. It consists in killing the tops with a herbicide and digging the crop ten days later after the tops have been thoroughly dried out. The results of their experiments are summarized by Bonde and Schultz as follows: "In 1943, 40 per cent of the tubers harvested from the green plots developed storage rot, whereas only 3 per cent rotted when harvested after the plants had been killed. In 1944, by killing the tops and digging 10 days later, tuber rot was reduced from 53.9 per cent to 3 per cent".

A "CROP FREE" PERIOD

Reducing the abundance of a plant disease by eliminating the crop for a period long enough to effect that result must frequently have been considered and has occasionally been suggested. Beginning in 1934 this method of disease control was tried with evident success in the Venice district of Los Angeles County, California (9). The disease in question was western celery mosaic. Continuous cropping has characterized the celery culture of this region for a long time. Plants of all ages thus occur on the same ranch or adjacent ranches at all seasons throughout the year. Prior to 1931, however, yields had been generally satisfactory. Beginning in that year yields throughout the district declined steadily. This decline was apparently due to a virus disease, western celery mosaic, which was so abundant that "all fields . . . were 100 per cent infected". This disease was either absent from or rare in the nearby celery-growing regions of San Diego, Orange and Ventura counties. This condition existed in spite of the fact that celery seedlings from the Venice district were used in these three counties. However, in none of them had continuous culture been practiced.

Acting on this field evidence it was suggested to the growers that a voluntary "celery free" period be established in 1934. This was done. The rule was made that no plant of *Apium graveolens* be grown in the field between July 31 and January 31 or in the glass

house between September 1 and October 30. Observation of this rule was made effective by regular inspections. In 1935 the declaration of a host-free period was authorized by the legislature of the State of California, since which time the practice has had a legal basis. In subsequent years the "celery-free" periods were somewhat changed. The effect on yield of the change in cultural practice is seen in the following table (9):

EFFECT OF THE CELERY-FREE PERIOD ON YIELD

<i>Year</i>	<i>Acreage</i>	<i>Yield per acre based on half-crates</i>
1930	700	1,026
1931	1,100	740
1932	1,500	661
1933	1,300	555
1934	900	311
1935	200	800
1936	950	926
1937	950	847

The years following the "celery-free" period are in bold faced type.

SOWING METHODS IN RELATION TO INCIDENCE OF GRAIN SMUTS IN EGYPT

In Egypt under perennial irrigation three methods of sowing grain are practicable and two are common. The method of sowing was found (5) to have a direct relation to the amount of smut in the ensuing crop.

The "herati" method consists in broadcasting seed on moist land and plowing it in. Under this method the soil in which the seed germinates is just moist enough to plow readily. The seed itself is buried at various depths, but, according to our authors, only that at an average depth of 8 cm. germinates effectively. The other common method, called "afir", consists in broadcasting on dry soil, harrowing the seed in and irrigating. By this method the seed is buried at a fairly uniform depth of 4 cm. and the soil is soaked immediately after. Examination of pairs of wheat fields showed that those herati-sown had consistently more flag smut than those afir-sown. Later experiments showed that there was two or three times more flag smut in herati-sown plots. It was also found that covered smut of barley (*Ustilago hordei*), bunt of wheat (*Tilletia foetens* (Berk & Curt.) = *T. laevis* Kuhn), and grain smut of millet and broom corn (*Sphacelotheca sorghi*) showed somewhat similar differences.

The authors concluded that the difference is the result of two factors, depth of sowing and soil moisture. In almost all cases (three exceptions in thirty) there was a progressive increase in disease with each increase in depth of planting. They believe that the difference is due to the fact that shallow planting shortens the susceptible stage of the host plant. While the effect of soil moisture appeared to be smaller than that of depth of planting, it was consistent throughout, wet soil decreasing the incidence of disease. Mud sowing, that is, planting on the surface of recently flooded lands, combines minimum depth and maximum moisture. This method (5, p. 55) "controls flag smut (*Urocystis tritici*) so efficiently that it offers an alternative means of disease control as efficient, or more efficient, than the usual means of seed disinfection or resistant varieties".

OVERPLANTING AS A MEANS OF REDUCING CROP LOSSES

Overplanting must be a very ancient method of securing a crop in spite of seedling diseases. Seldom, however, does it seem to have been emphasized in scientific literature¹. This technique was used to assure stands of sweet corn (*Zea mays saccharata*) in New York and New England during the outbreak of bacterial wilt (*Phytoplasma stewartii*) in 1932 and 1933.

Pool in 1926 (10) called attention to this means of reducing losses caused by stem rot of sweet potatoes (*Fusarium batatis* and *F. hyper-oxysporium*). In susceptible strains of the Jersey and Porto Rico varieties he suggested the use of two or three plants, instead of one, per hill. He found that even during the most virulent period (July and August) the disease seldom killed all three plants, and an almost perfect stand was obtained on plots where 17% and 46% of the single plants were destroyed.

In the Carolinas it has been the custom ever since the first outbreak of downy mildew of tobacco (*Peronospora tabacina*) to plant about double the bed space formerly used.

¹ Unless, indeed, one admits in this category the old rhymed formula for planting corn:

"One for the blackbird
One for the crow
One for the cut worm
And two to grow."

Published with some slight change of text by Morgan and Gale Tudury, "Folklore of the Farm", *Country Gentleman* (March, 1944), p. 12.

"SYRINGING" FOR THE CONTROL OF DOWNY MILDEW

"Syringing", the washing of plant parts with a spray of water under pressure, is a very old method of dealing with red spider in greenhouses. It is seldom recommended for the control of fungi, although Yarwood states that some greenhouse operators have observed that rose mildew was checked by the heavy syringing with water used to control red spiders. This seems to be another of the important facts which are common knowledge in the trade but not found in scientific literature. Syringing as a means of controlling powdery mildews of various species (*Euonymus* mildew (*Oidium euonymi-japonici* (*G. arcangeli*) Sacc.) on *Euonymus japonica* L.; rose mildew (*Sphaerotheca pannosa* (Wallr.) Lév.) on *Rosa* sp., variety Dorothy Perkins; bean mildew (*Erysiphe polygoni* DC.) on *Phaseolus vulgaris* L. variety Pinto; cucumber mildew (*Erysiphe cichoracearum* DC.) on *Cucumis sativus* L.; and barley mildew (*Erysiphe graminis* DC.) on *Hordeum vulgare* L. on five different hosts was tested by Yarwood (16). In every case there was a marked reduction in the infection. The important fact in this seems to be the inability of these fungi to regenerate from haustoria.

GROWING NEW ROOT SYSTEMS BY SOIL BANKING

Citrus growers in Florida have for many years practiced banking soil about the bases of the trees as winter approaches. In case of a serious freeze trees so protected are not killed to the ground. The body of uninjured tissue makes possible rapid "rejuvenation" of the tree the following summer. Observations resulting from this practice enabled Rhodes (11) to develop a method of restoring trees which had been nearly girdled by root rot (*Phytophthora parasitica*). It consists merely in banking the soil around the bases of attacked trees, thus stimulating the growth of new roots. In many cases the new root system is adequate to sustain the tree for a long period. This method of stimulating the production of new root systems was also found readily applicable to the so-called Australian pines (*Casuarina* spp.) attacked by *Clitocybe* root rot (*Clitocybe tabescens*). Rhodes further states that it is advisable to treat the tree before banking by cutting away the diseased bark and roots. In extreme cases a well of bricks is laid around the bases of the trees, and this is filled in with sand to a point well above the highest excised bark.

CONTROL OF CHLOROSIS IN AMERICAN GRAPES
BY THE USE OF EUROPEAN STOCKS

American (*labrusca*) varieties of grapes, particularly Concord, showed serious chlorosis when grown in the north central counties of Utah. The chlorosis was apparently caused by the soil having a high lime content which interferes with normal utilization of iron. Wann (15) notes that "Chlorosis of grapes became a major problem in southwestern Europe in the later years of the 19th century where *labrusca* stocks were introduced to combat phylloxera". This was so serious as to result in numerous crop failures and to prevent successful propagation of these stocks in many areas. Apparently, *vinifera* stocks have the capacity to secure the necessary iron from soil in which *labrusca* stocks became chlorotic.

Acting on this information he tried the experiment of grafting Concord and other *labrusca* varieties on *vinifera* stocks which had shown no chlorosis. "The results of these grafting experiments demonstrate that Concord scions grafted on certain *vinifera* stocks will develop into thrifty vines and will remain practically free from chlorosis, even when grown in a soil that produces severe chlorosis in Concord cuttings".

"DROWNING" SCLEROTIA

Deep plowing is often recommended as a means of preventing the fruiting of over-wintering sclerotia. Brooks (2), working with *Sclerotinia sclerotiorum* in Florida, found that this method gave poor control because all sclerotia were not buried to the required depth (more than three inches). Ninety per cent of the sclerotia were destroyed by flooding for six to eight weeks during the summer.

DIGESTION OF SCLEROTIA BY SHEEP

In the Salt River Valley of Arizona, lettuce is a fall, winter and spring crop. *Sclerotinia sclerotiorum* (Lib.) has caused heavy losses. When the crop is harvested, obviously diseased heads are left in the field. On these decaying heads large numbers of sclerotia develop with resulting infection of subsequent crops. Since sheep are sometimes pastured on lettuce fields, an experiment was conducted to determine the viability of evacuated sclerotia (3). Of 16,000 sclerotia fed to sheep, 94.6% were digested. Less than 1% of those evacuated whole were viable. The maximum period of

evacuation of living sclerotia by sheep proved to be four days. Under these conditions, pasturing sheep on infected lettuce fields after harvest, followed by a quarantine period of four days, should result in marked reduction of infective material. The results reported for *Sclerotinia sclerotiorum* (3) differ markedly from those of *S. rolfsii* (7). The latter investigators found that from 10% to 22% of the sclerotia consumed by sheep were recovered whole. These showed a viability of 1.6%. Brown suggests that this difference may be due to the large size of the sclerotia of *S. sclerotiorum*.

INJECTIONS

Injection of chemicals into host plants for the purpose of slowing or preventing growth of parasitic fungi has been tried occasionally over a long period. A recent report indicates some success in controlling apple mildew (*Podosphaera leucotricha*) by injection of apple shoots with a solution of sodium thiosulfate (12).

Howard reported in 1941 (4) that the effect of the toxin produced by *Phytophthora cactorum* in maple trees could be antidoted by injection of 0.5% di-hydro-chloride salt of di-amino-azo-benzine plus a solvent and a penetrant. "In excess of 350 confirmed trees, naturally infected by the bleeding canker fungus, have been injected with the antidoting chemical, and have subsequently exhibited stoppage of bleeding and marked improvement in vegetative growth. Whether the trees have been cured in any absolute sense remains to be seen, but the results indicate some possibilities to be explored in the practical control of plant disease."

In 1944 Stoddard (14) announced the control of x-disease of peach by injection with p-aminobenzenesulphanilamide. The seedlings had been inoculated by budding and the chemical introduced through the cut upper end of the main stem. Maltose and dextrose added to the solution reduced the injurious effect on the host without preventing its effect on the virus.

PRODUCTION OF DISEASE-FREE SEED IN SEMI-ARID AREAS

Seed production in semi-arid regions of the western states is an important industry. Various factors have entered into its development. One is that seed production under these conditions is free from certain serious seed-borne parasites. The possibility of this fact in relation to disease control was suggested nearly 40 years ago

by Whetzel (8, p. 15). Obviously, then, a discussion of this means of reducing disease will hardly be expected under the title of this article. However, this important method of disease control is so little recognized by those not directly interested in the crops concerned that a brief restatement of the factors involved seems warranted.

Twenty years ago L. K. Jones (6) tested a large number of samples of pea seeds. He found that "seed produced in the semi-arid seed-growing areas of the western United States was practically always free from fungous infection, while a large percentage of the seed produced in eastern North America was more or less infected with *Ascochyta* spp. and other fungi".

The situation as regards beans has recently been summarized (8) with reference to California-grown seed. The writers refer first to the well known fact that two of the most serious diseases of beans are seed-borne. These are bean anthracnose caused by *Colletotrichum lindemuthianum* and blight caused by several species of bacteria. In both cases the disease-producing agents are located within the seed coats and are not destroyed by any practicable seed treatment. The writers then call attention to the close relation between the spread of these diseases in the field and the presence of free moisture. Finally, they point out the climatic conditions under which disease-free seed can be produced "even though the original seed be contaminated with the causal organisms".

Much study has been given to diseases of beans in Louisiana and particularly to the use of seed from semi-arid areas. On the basis of experience in Louisiana, Plakidas (8, p. 15) points out that "it is . . . not enough to have seed that is *almost* blight-free. To get complete protection against blight it is necessary to plant seed which is *entirely* blight-free. . . ." A corollary is that it is advisable to guard against using land that was planted to beans the previous season and to avoid planting beans adjacent to a bean field which may bear an infected crop.

NOTE

After the foregoing had been in the editor's hands several months our attention was called by Dr. G. S. Chester to the following unusual note. It records the use of stagnant water as a fungicide.

So far the sole method of controlling the "iliau" disease of sugar cane (caused by *Gnomonia iliau*) has been the abandonment and

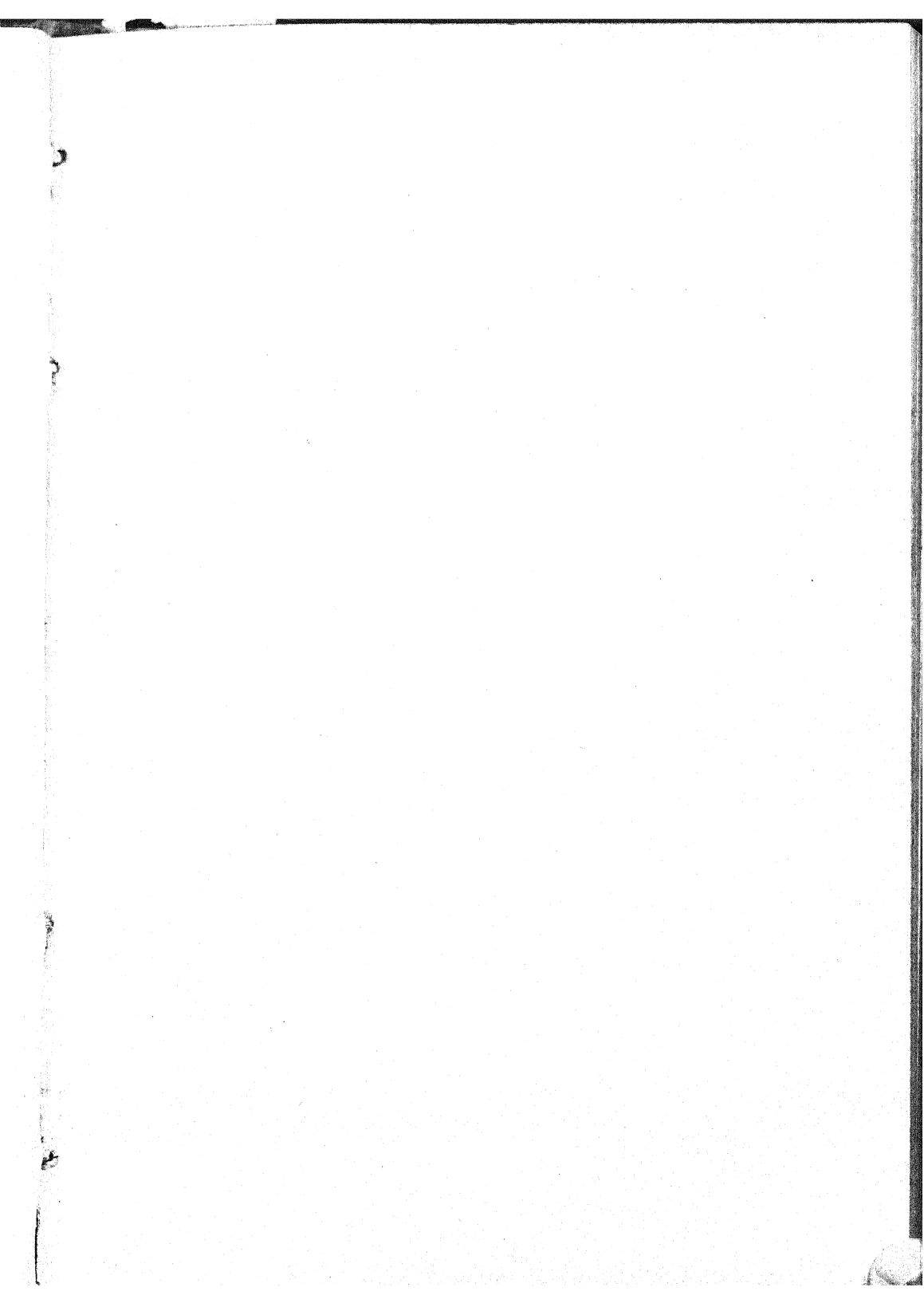
burning of the affected areas. Where the infection is not severe the writer's* tests have shown that the periodical application of "black water" (dark colored stagnant water from virgin forests, saturated with colloiddally suspended humic acids) is immediately lethal to the fungus, completely restoring the vitality of the diseased canes within a few days. Freise adds that "it must be doubted whether it is applicable in many cases."

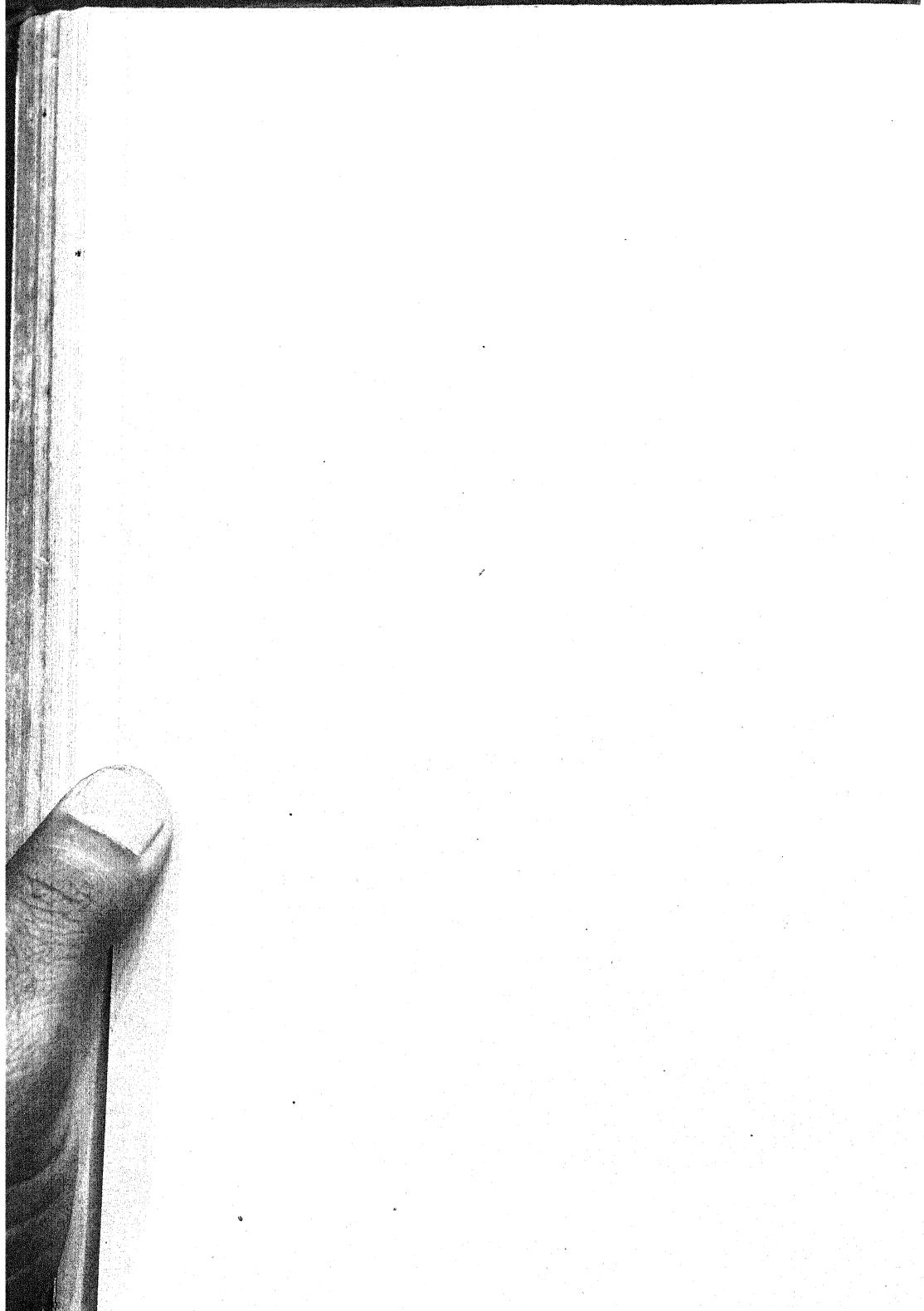
*Freise, F. W. Cane diseases and plagues in Brazil. Facts about sugar, 25: 613-614. 1930.

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THE MOVEMENT OF MATERIALS INTO PLANTS PART II. THE NATURE OF SOLUTE MOVEMENT INTO PLANTS

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INTRODUCTION

Many investigations have been recorded on limited aspects of the movement of solute into plants and plant cells. This treatise is an attempt to review and integrate certain evidence on migration of solute into plants, based as far as possible on current definitions and principles of physics and physical chemistry and in accord with a general scheme of treatment presented in Part I of this study.

THE THERMODYNAMIC PRINCIPLES OF DILUTE SOLUTIONS: THE
ESCAPING TENDENCY OR FREE ENERGY OF THE COMPONENTS

Applying the second law of thermodynamics to a dilute solution¹, we have, for either the solvent or the solute component, the partial differential relation

$$\left(\frac{\partial \bar{f}}{\partial p}\right)_{T,N} = \bar{v} \quad (1)$$

in which \bar{f} is the partial molal free energy; p is the pressure; \bar{v} is the partial molal volume, equal to the specific volume (or the reciprocal of the density) of the component at any particular temperature times its molal weight; the subscript T denotes constancy of temperature, and N constancy of composition. Solution of this mathematical expression yields the integral

$$\int_{f^0}^{\bar{f}} d\bar{f} = \int_{p^0}^p \bar{v} dp. \quad (2)$$

For dilute solutions, in which the pressure difference $p - p^0$ is small, \bar{v} may be regarded as constant and equal to \bar{v}^0 , the partial molal volume of a constituent of either component in a solution, in its reference or standard state. Hence, as an approximate equation we have

$$(\bar{f} - \bar{f}^0) = \bar{v}^0(p - p^0). \quad (2a)$$

By rearrangement of the terms in equation 2a, and to include, in the terms defined below, both the effects of those influences which increase and those which decrease the partial molal free energies of the constituent components of a solution, the approximate general equation to be used in this treatise is obtained, namely

¹ When the phrase "dilute solution" is used, it is meant that the principle or expression as stated approaches complete validity as the dilution is indefinitely increased. It becomes then a matter of experiment to determine to what concentrations such an expression may be regarded as valid within certain limits of permissible error. The degree of departure in "concentrated solutions" frequently depends on numerous factors.

$$\mp (p - p^\circ) = \frac{\pm (\bar{f} - \bar{f}^\circ)}{\bar{v}^\circ (1.013 \times 10^9)} = F \quad (2b)^2$$

in which \bar{f} is the partial molal free energy of a constituent of either component in solution, in a given state other than the reference state, in ergs; \bar{f}° is the partial molal free energy (44, p. 88) of a constituent, of either component in solution, in its reference state, in ergs; \bar{v}° is the partial molal volume of a constituent of either component, in the reference state, in liters; p° is the pressure on the solution in the reference state, in atmospheres; p is the pressure on the medium in the given state, necessary to make \bar{f} equal to \bar{f}° , in atmospheres; and F is the specific free energy of a constituent solute or water in solution. The reference states are: for solvent, that of pure water; for solute, that of a constituent solute species in solution at infinite dilution. Since the partial molal volume of a constituent, of either component, in the reference state is a constant (see footnote 1), it is evident from equation 2b that a specific free energy F (defined later), as well as the pressure difference $p - p^\circ$, is a measure of the quantity $(\bar{f} - \bar{f}^\circ)$.

The dimensional relationship between the quantities expressed by equation 2b may be outlined as follows:

$$F = \mp (p - p^\circ) = \frac{\pm (\bar{f} - \bar{f}^\circ)}{\bar{v}^\circ}$$

For $p - p^\circ$, the force per unit area = $m \times \frac{L}{t^2} \times \frac{1}{L^2} = \frac{m}{Lt^2} = mL^{-1}t^{-2}$,

where force = mass \times acceleration = $m \times \frac{L}{t^2}$ and area = L^2 . For

$\frac{(\bar{f} - \bar{f}^\circ)}{\bar{v}^\circ}$, the free energy difference or work per unit volume

= $\frac{mL^2}{t^2} \times \frac{1}{L^3} = \frac{m}{Lt^2} = mL^{-1}t^{-2}$, where energy difference or work = force

\times distance = $\frac{mL}{t^2} \times L = \frac{mL^2}{t^2}$ and volume = L^3 .

² This approximate, isothermal relation is applicable for dilute solutions on which any applied pressure is relatively small such that \bar{v} (the partial molal volume of a constituent of either component, in a given state other than the reference state) may be regarded as constant and equal to \bar{v}° (the partial molal volume of the constituent component under consideration, in its reference or standard state). Within the pressure limits normally occurring on the aqueous solutions of plants, the partial molal volume of either a solute species or the solvent in a given state may be assumed to equal, as a first approximation, its partial molal volume in the reference state. It may be noted that while the escaping tendency of a pure substance must increase with the pressure, this is not true for a constituent component in aqueous solution, where \bar{v} may be negative.

The Free Energy of Water in a Solution: For the solvent water in any dilute solution, the free energy equation 2b is particularly expressed by the approximate relation

$$\mp P = \mp (p - p^\circ) = \frac{\pm (\bar{f}_1 - f_1^\circ)}{\bar{v}_1^\circ (1.013 \times 10^9)} = F_1 \quad (3)$$

in which P is the osmotic pressure (15a). Here the reference state of the solvent is that of pure water. The partial molal volume of the water in an infinitely dilute solution \bar{v}_1 approaches the molal volume of pure water and may therefore be expressed by the constant v_1° at any temperature. For example, v_1° at 25°C. equals the specific volume of water (the reciprocal of the density) at this temperature multiplied by the molal weight of water or $v_1^\circ = 1.00294 \times 0.01801 = 0.01806$ liter. The pressure relationships of a dilute solution are further expressed by the equation

$$p - p^\circ = k \times \frac{N_2}{v_1^\circ} \times RT = F_1 \quad (4)^3$$

in which N_2 is the number of mols of solute in 1 mol of solvent; v_1° is the molal volume of the pure solvent, water; R is the appropriate gas law constant, here, 0.0821 liter-atmosphere per degree; T is the absolute temperature (see 44, p. 236). The pressure relationships of a dilute solution may also be expressed in terms of the solute concentration by the equation

$$(p - p^\circ) = kCRT = F_1 \quad (5)^3$$

in which C is the volume molar (molarity) or, better, the weight molar (molality) concentration of a constituent solute species in the solution⁴; R is the appropriate gas law constant, here, 0.0821 liter-atmosphere per degree; and T is the absolute temperature. In equations 4 and 5, the specific free energy of the water molecules F_1 is

³ If this equation of van't Hoff is used to define the specific free energy and concentration relations of an ideal solution, then departure from the ideal behavior must be interpreted with respect to such factors as are likely to influence the osmotic "solute" specific free energy (15a). He introduced a coefficient, k , into the equation, to correct for all types of deviation from ideal behavior without recourse to their origin. The osmotic "solute" specific free energy may be modified by the degree of dissociation of the molecules of the solute into constituent ions and by hydration, in aqueous systems. Further, corrections may be applied for non-solvent volume and for forces of attraction between particles in solution (15a, footnote 6). Compare the use of solute activities.

⁴ In dilute solutions the two methods of expressing the composition are essentially identical. One concentration may be converted into the other when the density of the solution is known (28a, pp. 137-139).

equivalent to the osmotic "solute" specific free energy F_s as defined in (15a).

The Free Energy of a Constituent Solute in a Solution: For a constituent solute species in any dilute solution, the free energy equation 2b is particularly expressed by the approximate relation

$$\mp (p - p^\circ) = \frac{\pm (\bar{f}_2 - \bar{f}_2^\circ)}{\bar{v}_2^\circ (1.013 \times 10^9)} = F_2 \quad (6)$$

Here, the reference state of the solute is that at infinite dilution. The partial molal volume of a constituent solute species \bar{v}_2 in dilute solution approaches its partial molal volume in the reference state at infinite dilution and may therefore be expressed by the constant \bar{v}_2° at any temperature. For example, \bar{v}_2° for the solute species KCl at 25° C. is approximately 0.02 liter (44, pp. 36-41).

The partial molal free energy difference of a constituent solute species in dilute solution from that in the reference state, is further represented by the approximate equation

$$(\bar{f}_2 - \bar{f}_2^\circ) = RT \ln \frac{C}{C^\circ} \quad (7)$$

$$= 2.303 RT \log \frac{C}{C^\circ} \quad (7a)$$

in which the difference in free energy $(\bar{f}_2 - \bar{f}_2^\circ)$ is expressed in ergs; R is the appropriate gas law constant, here, 8.316×10^7 ergs per degree; T is the absolute temperature; and C is the volume molar (molarity) or, better, the weight molar (molality) concentration of the solute species in the solution. Combining equations 6 and 7a, the following relation is obtained:

$$F_2 = \frac{2.303 RT}{\bar{v}_2^\circ (1.013 \times 10^9)} \times \log \frac{C}{C^\circ} \quad (8)$$

Since R , expressed in ergs per degree, is 8.316×10^7 , equation 8 becomes

$$F_2 = \frac{(2.303) \times (8.316 \times 10^7) T}{\bar{v}_2^\circ (1.013 \times 10^9)} \times \log \frac{C}{C^\circ} \quad (8a)$$

$$\text{or} \quad F_2 = \frac{(2.303) \times (0.0821) T}{\bar{v}_2^\circ} \times \log \frac{C}{C^\circ} \quad (8b)$$

Because C° approaches zero and $(\bar{f} - \bar{f}^\circ)$ approaches infinity at infinite dilution F_2 is also infinite with reference to water. Therefore, the specific free energy values for any particular solution phase

is without experimental significance. As a consequence, it is necessary to estimate the difference in specific free energy of the constituent solute species between any two solution phases *i* and *e*. This is expressed by the equation

$$(F_{2i} - F_{2e}) = \frac{(2.303) \times (0.0821) T}{\bar{v}_2^{\circ}} \times \log \frac{C_i}{C_e} \quad (9)$$

From equations 3, 4 and 9 it is obvious that the difference in specific free energy between two solution phases for either the solvent or any constituent solute species may be computed one from the other.

In dilute solution, the approximate (for limitations, see 44, pp. 319-323) isothermal value of *C* for any known constituent electrolyte (solute) species may be computed from the specific conductance of the solution through the relation

$$\Lambda^{\circ} = \frac{1000 L}{C} \quad (10)$$

in which Λ° is the equivalent conductance of the electrolyte species in the reference state at infinite dilution; *L* is the specific conductance of the electrolyte species in the solution in the given state, in mhos per centimeter⁵; and *C* is the concentration of the electrolyte species in equivalents per liter of solution^{5a}.

Since we have to deal with solutions in which the effective concentration is not closely given by the number of mols per liter of solution (or per 1000 g. of water), but is modified by interaction between particles in solutions (18), it may be preferable to include these effects in the concentration value, *i.e.*, to use activities of a constituent solute, as defined by Lewis and Randall (44, chap. 22), rather than concentrations. The fundamental equations relating to the escaping tendency of a solute species from a solution or between two solution phases presented hereinbefore will be modified to read:

$$(\bar{f}_2 - \bar{f}_2^{\circ}) = RT \ln \frac{a}{a^{\circ}}, \quad (11, \text{ see } 7)$$

⁵ In dilute solutions where Kohlrausch's Law of independent migration of ions is valid, the specific conductance of many agricultural waters or biological solutions composed essentially of strong electrolytes, when expressed in $\frac{(\text{mhos})}{(\text{cm.})} \times 10^4$, directly approximates the sum of the cations or the anions expressed as milli-equivalents per liter (m.e./l.) of solution; $\times 70$ approximates the concentration in parts per million (p.p.m.).

^{5a} In solutions in which the deviation of Λ (the equivalent conductance of the electrolyte species in the given state) from Λ° due to the concentration variant is experimentally significant, it may be essential to recompute the value of *C*. Then, the value of Λ derived from the approximated primary value of *C* is used with the experimental value of *L* to calculate a secondary, more valid value for the concentration of the electrolyte species.

or, since $a^\circ = 1$ (by definition),

$$(\bar{f}_2 - \bar{f}_2^\circ) = 2.303 RT \log a. \quad (12)$$

Further,

$$F_2 = \frac{(2.303) \times (0.0821) T}{\bar{v}_2^\circ} \log a \quad (13)$$

and

$$(F_{2i} - F_{2e}) = \frac{(2.303) \times (0.0821) T}{\bar{v}_2^\circ} \log \frac{a_i}{a_e} \quad (14, \text{ see } 10)$$

The activity a , or the effective concentration, may be obtained by correcting the concentration C , the latter expressed in molality, by means of a coefficient characteristic of the solute at a given molality and temperature (44, pp. 328, 329). Thus, $a = \gamma C$, in which γ is the activity coefficient. Activity coefficients of typical electrolytes are given in standard physical chemistry texts (44, pp. 344, 362). Similar values are reported for non-electrolyte (44, p. 288).

The movement of solute into plants generally involves highly ionized salts, and it is therefore advantageous to analyse the free energies of solute with respect to individual ions, both anion and cation, in the various phases of the system. Analysis may be made on the basis of molalities, or preferably activities, of the constituent ions just as was done for a constituent solute species. Another advantage accrues from this means of approach. It permits a free energy analysis in and between mixed electrolytes, even containing two or more valence types. This approach involves the additional principle of ionic strength. The ionic strength, μ , of a solution of strong electrolytes is defined as one half the sum of the quantities obtained by multiplying the stoichiometric molality of each ion by the square of its valence (or charge), *i.e.*,

$$\mu = \frac{(\text{molality} \times (\text{valence})^2)}{2} \quad (15)$$

In dilute solutions the activity coefficient γ of a given strong electrolyte is the same in all solutions of the same ionic strength. Further, it may be assumed that in dilute solution, the activity coefficients of any ion depends solely upon the total ionic strength of the solution (44, p. 380). Tabulation of the activity coefficients of individual ions at various values of the ionic strength have been compiled from available data (44, p. 382). The activity coefficients of $(\text{H}_2\text{PO}_4)^-$ may be computed from the equation

$$-\log \gamma_{(\text{H}_2\text{PO}_4)^-} = 0.5 \sqrt{\mu} \quad (16)$$

Application of these principles to a biological system has been made by Zscheile (81).

THEORETICAL ASPECTS OF SOLUTE MOVEMENT INTO PLANTS

The systems with which we have to deal, Figure 1, are aqueous systems, each essentially of two phases separated by a differentially permeable membrane—both typical osmometers. They are similar

DIAGRAM OF TWO POSSIBLE PLANT SYSTEMS FOR SOLUTE MOVEMENT

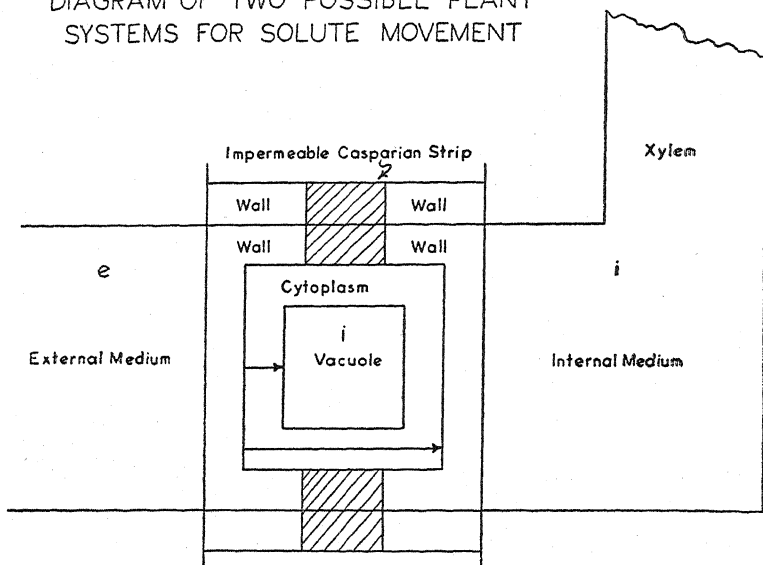


FIG. 1. Diagram of two possible plant systems for solute movement. Two imperfect osmometer systems are indicated: first, the simplified integrated system, external medium (water, nutrient solution, or soil medium)—membrane (endodermal cytoplasm or protoplasm of a comparably structured cell series)—internal medium (xylem solution, etc.); and second, a reference cell, external medium (nutrient solution or intercellular solution)—membrane (cytoplasm)—internal medium (vacuolar solution).

to the systems relating also to the movement of water. Deviations from the ideal system have been presented in Part I. Solute flux is a process of diffusion involving at one stage the movement of this component through or across the differentially permeable cytoplasm, including its limiting surfaces. The relative impermeability of the cytoplasm may regulate the rate of influx of a constituent species of solute. The fundamental principle underlying the movement of solute, definitions and theoretical evaluation of the constitu-

ent free energies tending to cause solute to move through the system are similar to those relating to water flow and also discussed in Part I of this study.

In the foregoing treatise on the movement of water into plants it was shown that the solvent component of the solutions of the plant osmometers always tends to flow with the direction in which its specific free energy decreases⁶, toward equality of escaping tendency. The rate of water flow was related to the permeability of any limiting region or regions, *i.e.*, the semipermeable membrane, and the specific free energy gradient (the space-rate of specific free energy change or inequality). The specific free energy which could be applied to tend to cause water to flow with the direction in which the concentration of water decreases (simple diffusion of water) was expressed in terms of an osmotic non-metabolic (*e.g.*, osmotic "solute" specific free energy) or metabolic specific free energy. The specific free energy which could be applied to tend to cause water to move against the direction in which the concentration of water decreases (accumulation of water) was expressed principally in terms of an osmotic metabolic specific energy.

Similar comprehensive statements can be made with regard to the flow of solute through the plant osmometers. A constituent species of the solute component of the solutions of the plant osmometers always tends to flow with the direction in which its specific free energy decreases, toward equality of escaping tendency. The rate of flow of a constituent solute is governed by the permeability of any limiting region or regions, *i.e.*, the differentially (including possible selectivity) permeable cytoplasm, and the specific free energy gradient (the space-rate of change of the free energy). (See section entitled "On the Rate of Movement of Materials into Plants".) The specific free energy which can be applied to tend to cause solute to flow with the direction in which the concentration of the solute species in solution decreases (simple diffusion of solute) is expressed in terms of a non-metabolic (*e.g.*, solute specific free energy) or metabolic specific free energy. The

⁶ Because the products of pressure and volume in the solution phases of the system are practically constant (compare 44, p. 204) in the process of flow of solute, the partial molal free energy F of the solute species, according to Lewis & Randall (44), is used. Contrast this with the free energy term " A " of Helmholtz necessarily employed in the discussion of water flow (see 44, pp. 156-159; 53, pp. 96, 97). The phrase "free energy" will be used hereinafter with the understanding that it refers to the partial molal free energy of the solute species.

specific free energy which can be applied to tend to cause solute (ion, ion pair or molecular species) to move against the direction in which the concentration of the solute species in solution decreases (accumulation of solute) is expressed principally in terms of a metabolic specific free energy (see discussion entitled "Metabolic Accumulation"). The metabolic free energy required to cause a solution component to move against the direction in which its concentration decreases is supplied through a restricted step or steps in oxidative metabolism characteristic of the organism.

On the basis of the principles here laid down, the various factors tending to cause the influx and efflux of solute are discussed according to the following outline, see figure 2 (compare 60) :

I. Flux of the solute species with the direction in which its concentration or activity decreases.

A. Simple diffusion of ions, ion pairs or molecules, including chemical double decomposition.

II. Flux of a solute species with or against the direction in which its concentration or activity decreases.

A. Flux not necessarily requiring solute-cytoplasmic interaction.

1. Donnan diffusion of electrolyte.

2. Pressure effects on solute.

B. Flux probably requiring some form of solute-cytoplasmic interaction.

1. Adsorption of ion pairs or molecules, and the exchange (exchange adsorption) of similar material between the surface layer of the micelle and the intermicellar dispersion medium. This accumulation is not directly related to oxidative catabolism. Exchange adsorption of substances involving interchange of solute species produced metabolically, may be termed "metabolic" exchange adsorption. That fraction exchanged exclusive of solute species produced metabolically, may be termed "non-metabolic" exchange adsorption.

a. Non-vital, that fraction not necessarily associated with the living system.

b. Vital, that fraction associated with the living system.

2. Metabolic accumulation, characterized by an overall unidirectional movement of electrolyte or non-electrolyte directly related to oxidative catabolism.

Specific Free Energies of Solute Defined: Specific free energies of solute, F_2 , are any action capacities of a constituent solute species in a solution, especially as they apply to the translation of particles of the substance in space, expressed in positive dimensions of $\text{mL}^{-1}\text{t}^{-2}$, e.g., energy per unit volume or pressure. A specific free energy of solute is numerically equal to the change in pressure on a medium necessary to render the escaping tendency of the solute species in a given state equal to that of the solute species in a reference state. The pressure difference is related to the free energy of a solute species in a dilute solution contained in either phase of the system through the approximate relation:

$$\mp (p - p^\circ) = \frac{\pm (\bar{f}_2 - \bar{f}_2^\circ)}{\bar{v}_2^\circ (1.013 \times 10^9)} = F_2 \quad (6)$$

In this treatise, specific free energies are used as measures of the differences in free energy of a solute species, due to any constituent influence tending to cause the solute to flow through the system, in order to deal with dimensions commensurate with those employed for osmotic influences tending to cause water to flow (15a). However, the action capacities may be expressed in fundamental energy units, e.g., ergs. For the units used in this dissertation, the conversion is accomplished through the relation $\pm (\bar{f}_2 - \bar{f}_2^\circ) = F_2 \times 1.013 \times 10^9 \times \bar{v}_2^\circ$, where F_2 is expressed in atmospheres; $(\bar{f}_2 - \bar{f}_2^\circ)$ in ergs; (1.013×10^9) is the necessary conversion factor; and \bar{v}_2° is a constant related to the particular solute species.

Metabolic Specific Free Energy of Solute Defined: In the plant systems (Figure 1), metabolic specific free energy (F_m) of solute is any possible action capacity maintained directly through metabolism of the living organism which can be applied to tend to cause a solute species to move unilaterally through or across the cytoplasm with or against the direction in which its concentration decreases, by modification of the free energy of the solute.

Non-metabolic Specific Free Energy of Solute Defined: In the plant systems (Figure 1), non-metabolic specific free energy (F_{nm}) of solute is any possible action capacity not directly maintained through metabolism of the living organism, which can be applied to tend to cause a solute species to move through or across

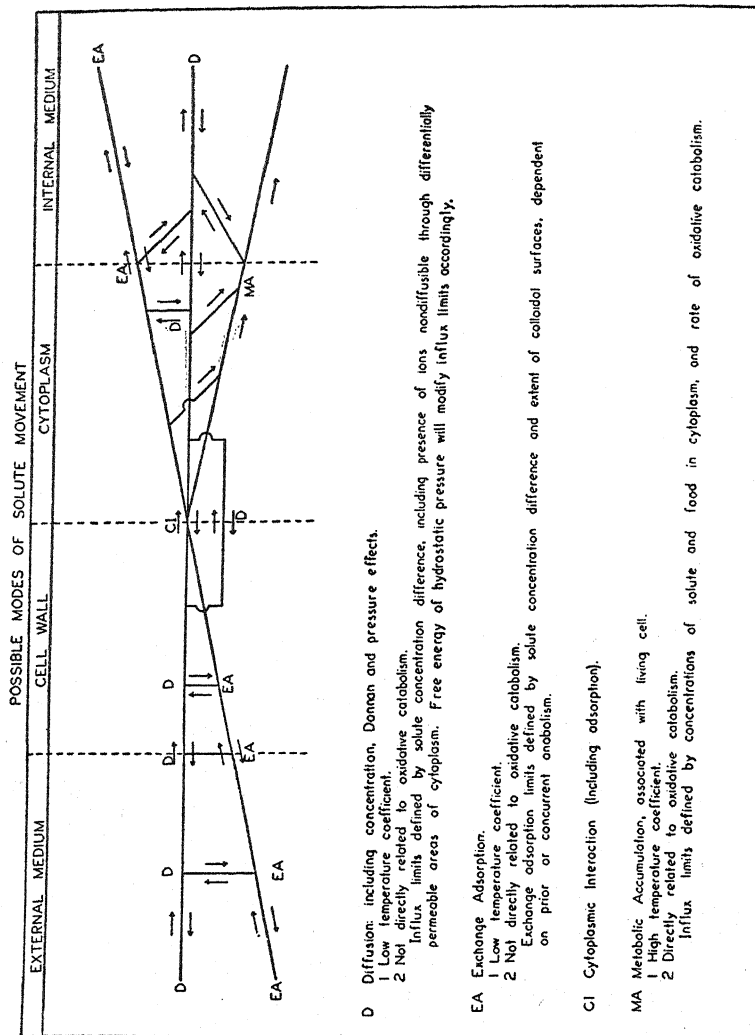


FIG. 2. Diagram of some possible modes of solute movement into plants. The figure is merely diagrammatic for a reference or integrated cell system. The slopes of the lines have no significance. Cytoplasmic interaction with solute may occur at the external surface of the cytoplasm or within the latter. The unidirectional nature of the metabolic accumulation process is indicated in the cytoplasmic region. It is open to question whether the influx of the solute species from the cytoplasm to the internal medium is with or against the direction in which its concentration in solution decreases.

the cytoplasm with or against the direction in which its concentration decreases, by modification of the free energy of the solute.

One typical influence which may lead to a non-metabolic specific free energy of solute is that due to the presence of a finite concentration of solute in solution (termed "solute specific free energy"). This is expressed through the relation

$$F_{S_2} = \frac{2.303 RT}{\bar{v}_2^\circ (1.013 \times 10^9)} \times \log \frac{C}{C^\circ} \quad (8)$$

derived from

$$(\bar{f}_2 - \bar{f}_2^\circ) = RT \ln \frac{C}{C^\circ} \quad (44, \text{pp. 214, 233}) \quad (7)$$

A second typical influence which may lead to a non-metabolic specific free energy of solute is that due to phenomena associated with colloidal surfaces, *e.g.*, adsorption.

Simple Diffusion Defined: Diffusion, in the general sense of the term, has been defined in Part I as the process whereby one substance moves into or through another in response to a quantitative difference of free energy, tending toward equilibrium of escaping tendencies. Simple diffusion is the diffusion of a substance with the direction in which its concentration (mols per liter, or better, mols per 1,000 gm. of water) in solution decreases, due to solute specific free energy.

Exchange Defined: Exchange is the process of substitution of one constituent for an equivalent constituent. Two types of exchange may be involved in the movement of solute in the system; first, chemical double decomposition, and second, exchange adsorption.

Accumulation Defined: Accumulation is the diffusion of a substance against the direction in which its concentration in solution decreases. Two forms of solute accumulation are manifested in plants; first, metabolic accumulation; and second, non-metabolic accumulation. Metabolic accumulation requires direct expenditure of metabolic specific free energy. Non-metabolic accumulation does not require direct expenditure of metabolic specific free energy. Several types of non-metabolic accumulation may take place in plants. One, Donnan diffusion, relates to ion movement and requires an interposed differentially permeable membrane through or across which some ions are relatively incapable of passage (29). A second type is characterized by the process of adsorption.

Adsorption Defined: Adsorption is the process whereby substances in solution in a dispersed system vary in concentration, generally an increase or an accumulation, at the surface of a colloidal micelle from that in the body of the intermicellar medium, irrespective of the mechanism by which this difference is brought about. Some types of force and chemical bond which may occur in adsorption phenomena are electrostatic bonds, polarization bonds, electron pair bonds, hydrogen bonds, and Van der Waal forces (40). "Exchange adsorption" is the process whereby solute species previously adsorbed on a colloidal micelle are interchanged with similar matter from the intermicellar fluid.

Absorption Defined: The term absorption has been generally used to mean the process whereby influx of materials occurs, irrespective of the mechanism by which this is accomplished. In a restricted sense this term has been limited to situations where equality of concentration of the substance (true solution) exists throughout a phase to distinguish it from adsorption where the concentration within a phase is non-uniform due to accumulation at surfaces of colloidal micelles included within the phase. The term sorption has been suggested as a general term, where the uniformity of concentration is not distinguished. In order to avoid any ambiguity, the terms influx and efflux are employed here to characterize the inward and the outward movements, respectively, of materials between solution phases of the plant systems.

Net Influx Specific Free Energy Defined: Similar to the definition outlined for the movement of water into plants, the net influx specific free energy of solute, NIF_2 , is the difference in action capacity between the algebraic sum of those specific free energies tending to cause solute to move into the reference system, and those tending to cause solute to move out of the system. The net influx specific free energy of solute is equal to the sum of the influx specific free energies diminished by the sum of the efflux specific free energies, i.e., net influx specific free energy = (Σ influx specific free energies) - (Σ efflux specific free energies) or

$$NIF_2 = \Sigma IF_2 - \Sigma EF_2. \quad (17)$$

Comparison Between Two Alternative Aspects of the General Process of Solute Flow into Plants: There are two alternative methods of viewing the solute relations in plants comparable with those discussed for osmotic (water) relations. Consider the process

in which a particular solute species moves from one phase to another through an interposed membrane or limiting surface, *i.e.*, solute (external) $\xrightarrow{\text{influx}}$ solute (internal). The measure of the tendency for the solute species to move is given by the difference between the free energy of the solute in the external phase and that in the internal phase of the system, $(\bar{f}_i - \bar{f}_e)$. If this difference is negative in sign, the solute will tend to move inward, as written. Thus, if it is desired to know whether or not the solute will tend to move across the interposed differentially permeable region, the quantity $(\bar{f}_i - \bar{f}_e)$ must be determined.

In practice, the free energies themselves are not determined, but the difference between the free energy in a given state and the free energy in a reference state. For solute, the reference state is frequently chosen as that at infinite dilution in the solvent water under otherwise standard conditions. If, by thermodynamic methods, the quantities $(\bar{f}_i - \bar{f}_i^\circ)$ and $(\bar{f}_e - \bar{f}_e^\circ)$ are determined, then the difference between these quantities is $(\bar{f}_i - \bar{f}_e)$.

As will become clear, there is an entirely equivalent method of measuring the tendency for the above process to occur. This method involves the measurement of the specific free energies of solute (F) in each phase of the system, distinguishing between those constituent influences which tend to decrease the free energy of the solute component in the given state with respect to that in the reference state $(-\Delta f)$, and those which correspondingly tend to increase the free energy (Δf) . Addition of a finite (yet small) amount of solute will raise the free energy of solute above that in the reference state. The presence of an adsorbent surface within a phase will tend to lower the free energy of the solute within that phase. Application of a pressure upon a solution will tend to increase the free energy of the solute, as well as the solvent, within a phase. This physico-chemical analysis of the specific free energies of solute involved in the system will be presented by means of equations which apply only for dilute solutions.

The specific free energy of solute is defined as equal, both dimensionally and numerically, to the difference in pressure on a solution necessary to make the free energy of the solute in the given state the same as that of the solute in a reference state at infinite dilution. For dilute aqueous solutions, a constituent specific free energy of solute F_2 was given by equation 6, *viz.*,

$$F_2 = \mp (p - p^\circ) = \frac{\pm (\bar{f}_2 - \bar{f}_2^\circ)}{\bar{v}^\circ (1.013 \times 10^9)} \quad (6)$$

Since the partial molal volume of a solute species at infinite dilution is a constant, isothermally, it is evident that the specific free energy F_2 is a measure of the quantity $(\bar{f}_2 - \bar{f}_2^\circ)$. For a single constituent influence tending to modify the free energy of the solute it follows that

$$F_i - F_e = \frac{\pm (\bar{f}_i - \bar{f}_e)}{\bar{v}^\circ (1.013 \times 10^9)} \quad (18)$$

where F_i and F_e are the specific free energies of solute related, isothermally, to constituent influences associated with the internal and external phases, respectively, of the system. As previously stated, $(\bar{f}_i - \bar{f}_e)$ is a measure of the tendency for a solute species to move across the membrane or limiting cytoplasm. From equation 18 it is apparent that the quantity $(F_i - F_e)$ is also a measure of the tendency for solute to move. A negative value for $(F_i - F_e)$ indicates a tendency for the solute species to move inward, *i.e.*, an influx of the solute.

A finite increase of solute concentration in dilute solution within a phase of the system ordinarily is accompanied by an increase in the free energy of the solute species. For solute, the change in free energy due to an increased concentration of the solute species in solution is approximately proportional to the increase in concentration, *i.e.*,

$$F_{(\Delta f)} = - (p - p^\circ) = \frac{(\bar{f} - \bar{f}^\circ)}{\bar{v}^\circ} = \frac{2.303 RT}{\bar{v}_2^\circ (1.013 \times 10^9)} \times \log \frac{C}{C^\circ} \quad (\text{see equation 8}).$$

On the other hand, the change in free energy due to the presence of an adsorbent in a phase of the system is proportional to the negative of the specific free energy, *i.e.*, $F_{(-\Delta f)} = (p - p^\circ) = \frac{-(\bar{f} - \bar{f}^\circ)}{\bar{v}^\circ}$.

The two equations, therefore, have the same form, but with opposite sign. If the specific free energies, numerically equal to the imposed pressures necessary to make \bar{f} equal to \bar{f}° , are designated by the symbol $F_{(-\Delta f)}$ for constituent influences lowering the free energy of the solute species (*e.g.*, an adsorbent, F_a or F_{nm}) and by the symbol $F_{(\Delta f)}$ for constituent influences raising the free energy (*e.g.*, additional solute, F_s), it follows that

$$(\Sigma F_{(-\Delta t)} - \Sigma F_{(\Delta t)}) = \frac{-(\bar{f} - \bar{f}^o)}{\bar{v}^o (1.013 \times 10^9)} \quad (19)$$

and for the two phases of the system, that

$$\begin{aligned} ((\Sigma F_{(-\Delta t)}) - (\Sigma F_{(\Delta t)}))_i - ((\Sigma F_{(-\Delta t)}) - (\Sigma F_{(\Delta t)}))_e \\ = \frac{-(\bar{f}_i - \bar{f}_e)}{\bar{v}^o (1.013 \times 10^9)} = NIF_2 \quad (20) \end{aligned}$$

Thus the expression $((\Sigma F_{(-\Delta t)}) - (\Sigma F_{(\Delta t)}))_i - ((\Sigma F_{(-\Delta t)}) - (\Sigma F_{(\Delta t)}))_e$ is also a measure of the tendency for the solute species to move. A positive value for this quantitative difference indicates a tendency for the solute to move inward, *i.e.*, an influx of solute. One hypothetical example of the use of equation 20 may be cited, *e.g.*, the tendency for solute to move inward,

$$NIF = ((F_{nm_i}) - (F_{s_i})) - ((F_{nm_e}) - (F_{s_e} + F_m)). \quad (\text{compare } 15a). \quad (20a)^7$$

In order to express these specific free energies in equations which can be subsequently discussed in relation to the possible consequent movement of water, an alternative scheme is presented. Here, as in the treatise on water flow, the difference between the algebraic sum of the action capacities tending to cause solute to move inward

⁷ It may be observed that the metabolic specific free energy factor tending to cause the component (solute here) to move inward in the system is designated as an influence tending to increase the free energy of the component in favor of the external phase. The over-all movement of the component against the direction in which its concentration, or activity decreases, is in a direction opposite to that in which a flow "normally" would occur, and is therefore associated with an apparent increase in free energy between the two phases. The law of tendency toward flow with the direction in which the specific free energy of the component decreases is maintained by asserting a complementary decrease of free energy within the cytoplasm, more than sufficient to compensate for the increase in question (44, pp. 120, 121). The oxidative catabolism experimentally required for metabolic accumulation is involved in this way. Data of Ulrich (78) have shown that under aerobic conditions the increased utilization of oxygen (O_2 involution) may measure the enhanced oxidative catabolism more adequately than the carbon dioxide production (CO_2 evolution). This is true because an unbalanced metabolic accumulation of the anion and cation supplied from a single salt species may lead to a modified respiratory quotient involving organic acid metabolism. Estimation of the change in concentration of a metabolite may be quite inadequate (34, 47). Under anaerobic conditions other means of measurement of oxidative catabolism must be sought. The measurement of total oxidative catabolism may be viewed as consisting of two complementary fractions, an intrinsic portion and an extrinsic portion. The latter is that fraction associated with a single variable under a given set of conditions, whereby the total is modified from that under a reference or control situation. However, exact interpretation of these two portions in terms of chemical or energetic changes related to treatment is difficult, since they are so intimately interrelated that probably they are mutually altered by the change of state.

and the algebraic sum of those tending to cause solute to move outward is a measure of the net tendency for the solute species to move inward across the cytoplasm or limiting region. The specific free energies of a solute species, regrouped in the equation of net tendency for solute flow (equation 17), are expressed in terms of the changes in the actual specific free energies of the solute species, due to existing action capacities related to the constituent influences in a phase of the system. Based on the relation $NIF = \sum IF - \sum EF$, the constituent factors, as expressed in equation 20, would be regrouped to read

$$NIF = ((\sum F_{(-\Delta f)})_i + (\sum F_{(\Delta f)})_e) - ((\sum F_{(\Delta f)})_i + (\sum F_{(-\Delta f)})_e) \quad (21)$$

APPLICATION OF THE NET INFLUX SPECIFIC FREE ENERGY EQUATION

The physico-chemical discussion of the free energies possibly involved in the movement of solute into plants or plant cells is of great theoretical interest. It presents a fundamental view point of the process of influx of solutes and its interconnected rôle in the movement of water⁸. Most of the factors involved in the flow of water have been estimated in physico-chemical terms. So far, no attempt has been made to estimate the latent specific free energies which tend to cause solute to move in the system. Experimentally, these may be difficult or impossible of realization. Certain computations have been made of the energy expended in net accumulation of solute (81; compare 64). These results were obtained from measurements of the relative activities of a solute species in the two phases of the system. Such figures are the result of the integrated effect of the flow of solute and water due to physico-chemical causes.

As stated hereinbefore, it is possible by means of equations 3, 4 and 10 to compute the difference in specific free energy of a solute species, between two solution phases, from that of the solvent. As a typical case, we may analyze the specific free energy of solute for the reference cell system outlined earlier for water flow. Assuming water equilibrium for the system: external medium—membrane (cytoplasm)—internal medium (vacuole) discussed as equation 39 in the treatise on water movement into plants, and a single salt species KCl at 25° C., we may find the theoretical difference in

⁸ A certain amount of water will probably move in direct association with solute, as water of hydration (47).

specific free energy of the constituent solute between the two solution phases. For the solvent, we had at water equilibrium,

$$\begin{aligned} \text{NIF}_1 &= \Sigma \text{IF}_1 - \Sigma \text{EF}_1 \\ \text{NIF}_1 &= (\text{Fs}_{11}) - (\text{Fs}_{1e} + \text{Fh}_1) \end{aligned} \quad (22)$$

$$0 = (16.6) - (4.0 + 12.6) \quad (22a)$$

For the solute at thermodynamic equilibrium, we have

$$\begin{aligned} \text{NIF}_2 &= \Sigma \text{IF}_2 - \Sigma \text{EF}_2 \\ \text{NIF}_2 &= (\text{Fm (or Fnm)} + \text{Fs}_{2e}) - (\text{Fs}_{21} + \text{Fh}_1) \end{aligned} \quad (23)$$

Since concentrations in mols of solute per liter of solution are employed here, Fs_{2e} and Fs_{21} can not be determined, but the difference between the solute specific free energies due to the constituent solute in the two phases can be computed. Equation 23 then becomes

$$\text{NIF}_2 = (\text{Fm (or Fnm)}) - ((\text{Fs}_{21} - \text{Fs}_{2e}) + \text{Fh}_1) \quad (24)$$

The quantity $(\text{Fs}_{21} - \text{Fs}_{2e})$ is obtained by means of equations 5 and 9. Knowing Fs_{11} and Fs_{1e} and using equation 5⁹, the solute concentrations in the two phases are found. Thus,

$$C_i = \frac{16.6}{0.0821 \times 298} = \frac{16.6}{24.45} = 0.678 \text{ equivalents of KCl per liter,}$$

and

$$C_e = \frac{4.0}{24.45} = 0.1635 \text{ equivalents of KCl per liter.}$$

Having found C_i and C_e , $(\text{Fs}_{21} - \text{Fs}_{2e})$ may be found from equation 9. Thus

$$\begin{aligned} (\text{Fs}_{21} - \text{Fs}_{2e}) &= \frac{(2.303)(0.0821)(298)}{0.02} \times \log \frac{C_i}{C_e} \quad (25) \\ &= 2.815 \times 10^3 (\log 0.678 - \log 0.1635) \\ &= 1.74 \times 10^3 \text{ atmospheres.} \end{aligned}$$

Setting the quantities representing $(\text{Fs}_{21} - \text{Fs}_{2e})$ and Fh_1 into the net influx equation for solute, at equilibrium

$$\text{NIF}_2 = (\text{Fm (or Fnm)}) - ((\text{Fs}_{21} - \text{Fs}_{2e}) + \text{Fh}_1) \quad (24)$$

$$0 = (\text{Fm (or Fnm)}) - (1.74 \times 10^3 + 12.6) \quad (26)$$

The lack of numerical equivalence between the quantities ΣIF and ΣEF , equal here to an efflux specific free energy of 1.75×10^3 atmospheres, indicates that another factor, or factors, are involved in the

⁹ Here, it is assumed that the isothermal temperature was 25° C. Because the specific free energy values were somewhat arbitrarily selected, although approximating experimentally observed quantities, and since data are lacking to justify the application of an osmotic "solute" specific free energy correction other than unity, it is assumed here, that this coefficient is equal to one (see footnote 3).

flow equation. This may be balanced through application of either an influx metabolic accumulation factor or an influx non-metabolic accumulation factor, or both, here equal to 1.75×10^3 atmospheres. Subsequent discussions will indicate that, generally, both influences are operative in the plant system.

In this treatise, the factors involved in the free energy of a constituent solute component, in either phase of the reference plant osmometers, are expressed in dimensions of $\text{mL}^{-1}\text{t}^{-2}$ (*e.g.*, atmosphere units) in accord with the dimensions measurable and used in connection with the constituent influences related to the escaping tendencies of water in the system. The free energy expended in the accumulation of solute, calculated from the experimentally observed difference in concentration or activity of the solute species between the external and internal phases of the reference systems, or the free energy released in oxidative catabolism associated experimentally with a particular solute accumulation, measured for example by the aerobic catabolism of carbohydrate (see 34, 47) or the change in CO_2 and O_2 relations of the system (see footnote 4), has been generally expressed in dimensions of mL^2t^{-2} (*e.g.*, erg or calorie units, where 1 calorie = 4.185×10^7 ergs). For the units used in these disquisitions, the dimensional interconversion may be accomplished through the relation

$$\pm (\bar{f} - \bar{f}^0) = F \times \bar{v}^0 (1.013 \times 10^9) \quad (27)$$

where F is expressed in atmospheres and $(\bar{f} - \bar{f}^0)$ in ergs. For the solute species KCl , \bar{v}_2^0 is approximately 0.02, expressed in liters (see page 129). In equations 25 and 26, the difference in specific free energy ($F_{s_{21}} - F_{s_{2e}} = 1.74 \times 10^3$ atmospheres), related to the existing difference in concentration of the constituent solute (*e.g.*, KCl) between the two phases of the reference system, could be expressed in dimensions of energy. Thus $(\bar{f}_i - \bar{f}_e)$ equals $(1.74 \times 10^3) (2.026 \times 10^7) \times 10^3 (2.026 \times 10^7) = 3.53 \times 10^{10}$ ergs or $\frac{(1.74 \times 10^3) (2.026 \times 10^7)}{4.185 \times 10^7} = 843$ calories. Similarly, in equation 26, F_m or F_{nm} could be expressed in dimensions of energy. Thus $(\bar{f}_i - \bar{f}_e)$ here equals -3.55×10^{10} ergs or -848 calories.

THE POSSIBLE MODES OF SOLUTE FLUX IN THE REFERENCE SYSTEMS OF THE PLANT

Numerous papers have been published reporting data on the movement of solutes between the plant organ and its bathing

medium. Digests of these researches have been made in the Annual Review of Biochemistry by various authors (22, 69, 82), in The Botanical Review (54) and elsewhere (5, 9, 31, 32, 33, 46, 47, 55, 56, 70, 73). In this section of this treatise certain experimental results will be mentioned to illustrate the use of the proposed outline for flow of solute into plant organs. The various modes of solute movement are intimately interrelated, but may be viewed separately for means of discussion.

Simple Diffusion: Simple diffusion was defined earlier as a movement with the direction in which the concentration of the solute species (ions, ion pairs or molecules) in solution decreases. The process is characterized by a low temperature coefficient and is not directly related to oxidative metabolism. Under certain conditions, however, the rates of flow by simple diffusion may be enhanced within the living organism through cytoplasmic streaming. Obviously, wherever a difference in concentration of a constituent solute exists within a solution or between two solution phases, the solute will tend to move toward equality of concentration, or better, toward equality of activity. Any influence which can modify the solute specific free energy may likewise modify the gradient of free energy and consequently the rate of flow toward thermodynamic equilibrium. A simple diffusion equilibrium could of course be altered through precipitation, or conversion into an insoluble form, or into a compound less capable of passage through an interposed membrane. Early investigators considered all movement of solute as merely simple diffusion (61, compare 77). Analyses of the vacuolar contents of certain large unicellular organs like *Valonia* (54, 82), *Nitella* (31, 37, 39, 81, 82), and certain Characeae (20, 82), and of exudates from plants (25, 36, 46, 47), showed that as a net result solute may flow against the direction in which the concentration of the solute species in solution decreases. Similar conclusions were drawn from analyses of composite expressed sap of various plant species (7, 16, 30, 34, 49, 69, 82), and changes in composition of a bathing medium in which plant tissues were cultured (46, 47, 64, 65, 73, 74, 75, 76).

Donnan Diffusion: A Donnan equilibrium (1, 18, 29) tends to arise whenever an ion or charged particle, *e.g.*, a colloidal micelle, is restrained in its movements in any way. The typical restraint is a membrane through or across which the charged particle can not

pass. According to this theory, for any pair of anions or cations, the following concentration ratios must exist at equilibrium between the two solution phases separated by the interposed differentially permeable membrane:

$$\left(\frac{[\text{Anions}]_i}{[\text{Anions}]_e} \right)^{1/Z_a} = \left(\frac{[\text{Cations}]_e}{[\text{Cations}]_i} \right)^{1/Z_c} \quad (28)$$

in which *i* and *e* are subscripts indicating phases internal and external to the membrane; and Z_a and Z_c are the valencies of the anion and cation, respectively (18).

This mechanism has been plausibly extended by Donnan (26) to include uncharged particles, in either phase, incapable of passage across the membrane. It is postulated that the uncharged particle reacts chemically with either positive or negative ions within its phase to form a charged "complex" which likewise is incapable of passage through the boundary. This second, neutral-particle type of equilibrium is theoretically sound.

Both forms of Donnan equilibria will tend to be approached in the biological systems of plants, provided the necessary assumptions are realized. The ionized-particle type, in association with simple diffusion, can not explain the salt balance found experimentally for *Valonia*, *Halicystis* and *Nitella* in their natural bathing media (see 54 and equation 28; compare 67). The neutral-particle type can not validly explain the net influx of solute until the "dissolved impermeable neutral substance" and the consequent "impermeable 'complex'" are isolated from the system, experimentally.

Pressure Effects: An increase of pressure, on or within a solution phase of an osmoscope, will increase the escaping tendency or specific free energy of both the constituent solutes and the solvent. Conversely, a tension will lower the specific free energy (for possible exception see 44, p. 213). In the section entitled "Application of the Net Influx Specific Free Energy Equation", it was indicated by equations that an internal positive net hydrostatic specific free energy contributed as an efflux tendency for solute. Under conditions where a negative net hydrostatic specific free energy arises (see 15a, Table I), this factor participates as an influx tendency. It therefore becomes clear that the tendency for solute to flow into a reference cell system is enhanced by plasmolysis. Similarly, for an integrated referency system, an increased tendency for influx of solute will obtain under actively transpiring conditions where the internal net hydrostatic specific free energy is negative.

Electrical and Thermal Effects: Electrical phenomena are undoubtedly connected with life processes, but although some speculations (46) have been made relative to their rôle in the movement of materials (solute and/or water) into plants, the cause and effect relations have not been elucidated. Likewise, any possible relationships between the movement of materials and temperature differences are obscure. Effects of the latter include modification of the specific free energies of the solute and solvent molecules of solutions, of the rate of metabolism and its consequent influences, and of the product of pressure and volume within a solution phase.

Since the solute balance in plant systems can not be explained entirely by the processes of simple diffusion, Donnan equilibria and pressure effects, other modes of solute flow must be also involved. These processes do not necessarily require any form of solute-cytoplasmic interaction. The modes of solute accumulation to be presented hereinafter probably require an interaction, at least as a primary step, between cytoplasm and solute at plasmatic interfaces. It may be noted that all the phenomena of surfaces occur not only at the obvious interfaces of the ectoplasm and endoplasm but throughout the colloidal substance (mesoplasm) of which the cytoplasm is composed. Cytoplasm is itself a heterogeneous system of more than one phase, and throughout this system, wherever there is a boundary surface between the dispersed phase and the dispersion medium, the surface phenomena may occur. One concept for solute-cytoplasmic interaction would include adsorption of the solute. Thus electrolytes and possibly non-electrolytes (depending on their presence in the external or internal media) may be interacted upon or adsorbed in this cytoplasmic region. Suggestions of various investigators that "absorption" (influx) is related to adsorption are probably involved in this way (74, 75, 76; compare 71). Robertson (64, 65) proposed a similar initial step, visualizing accumulation as taking place from a plant surface-medium higher in solute concentration than that in the bathing solution. He considers this concentration of the constituent solute to be constant under a given set of conditions.

Adsorption: Adsorption is the process whereby substances are concentrated at an interface or on a surface regardless of the mechanism whereby this localized accumulation occurs. The flow of solute by this mode of movement is accomplished through the

lowering of the free energy of the adsorbed solute particles. The process is characterized by a low positive, or even a negative, temperature coefficient. It is not directly related to oxidative catabolism. The adsorption equilibrium limits are defined, first, by the concentration of the solute within the dispersion medium; and secondly, the extent of the colloidal surface. Adsorption is possible by both the non-living structural matter and the living body of protoplasm of the organ. Non-vital and vital adsorption may therefore be distinguished, related to these two colloidal surface fractions. The extent of the colloidal surface of the living protoplasm is dependent on prior or concurrent anabolism; that of the non-living material may be related to prior anabolism. Adsorption is a thermodynamic equilibrium process. Like chemical double decomposition in true solution, adsorption involves continued displacement of material accumulated at the surface of a colloidal micelle for similar material in the body of the intermicellar medium of a dispersed system. This exchange adsorption occurs between particles of the same solute species, or if more than one species is involved, between similar particles of the various species comprising the solute component. When the solute species is produced through metabolism of the organ, the exchange adsorption may be considered as metabolic. In all other cases the interchange is non-metabolic whether related to surfaces of vital or non-vital material.

"Pseudo-adsorption" may be involved in the general process of solute movement (29). Here, the adsorption *per se* may be followed by a chemical change in the material which has been adsorbed, the chemical change being of such a nature as to inhibit the reversal of the adsorption process.

In early studies with abscised barley roots (34), losses of solute from inactive or moribund cells were recognized to occur. The probability was also present in experiments with abscised roots completely immersed in solution, of slight loss of solute through exudation from the cut surfaces. Re-influx, however, would occur to actively metabolizing cells. Furthermore, although under favorable metabolic and experimental conditions, a large net inward movement of salt occurred, the possibility of a simultaneous, dynamic two-way movement of solute between the root and the external medium was not excluded. Hoagland and Davis (39), using *Nitella*, reported anion exchanges of large magnitude. Other re-

sults (mainly unpublished, see also 34, p. 498) indicated some exchange of ions between abscised root systems and the nutrient medium. These exchanges particularly involved H^+ for K^{+10} ; HCO_3^- for Br^{-10} ; and Cl^- for Br^{-10} . (Also compare 4, 46, 50 and 23 using other types of tissues.) Some investigators (6-15, 45-47) have suggested various ion exchange mechanisms as prominently involved in the net accumulation of solutes by plant cells. Attention has been drawn (34) to the insufficiency of these mechanisms in themselves to explain the unidirectional process of salt accumulation from the external to the internal phases of the reference system of plants. Steward (70) has reported an exchange of cations, using excised discs of potato tissue, proceeding to approximately the same extent in either the living or the dead system. He distinguished definitely, however, this exchange of ions which is of small magnitude, from the "major" process of accumulation of solutes by metabolizing cells which was characterized by a net influx of K and Br ions in approximately equivalent quantities. Jenny and Overstreet (41) have presented results of experiments on decapitated barley plants that indicated an exchange of cations between roots and either culture solutions or colloidal suspensions accompanying the process of metabolic accumulation of solute. Further, it was postulated that direct "contact exchange" of cations occurs, under certain conditions, between soil colloids and root surfaces without preliminary entry of the ions into the dispersion medium or soil solution. Successive exchanges (in point of space) may be accomplished by surface migration phenomena (43, 63).

In the experiments with abscised barley roots immersed in salt solutions (34), exchanges of the cation K for Ca, Mg or Na, of important magnitude in relation to the net K accumulated, based on studies of expressed sap, were not evident during periods of salt accumulation. These results are in accord with more recent ones (40, 41). Further evidence by the latter investigators, however, indicated that "barley roots have a tendency to yield measurable amounts of K to certain electrolyte solutions." This result was obtained for the potassium ion by using radioactive and non-radio-

¹⁰ Although the possibility of exchange with metabolically formed organic cations and organic acid anions as such exists, the validity of this hypothesis has not been established (15, 51). Increase of hydrogen ion in the external medium is frequently inadequate to explain the excess influx of cation over anion by cells (35).

active isotopes associated with various anions. It was stated that "the intake of ions is not a unidirectional process; ions of the same species may move into the root and out of the root at the same time".

The net inward movement of ions ordinarily measured was recognized to be associated with certain metabolic activities as noted above. It remained to be determined whether the limited outward movement was likewise directly dependent upon the same metabolic factors required for the ionic accumulation, previously observed. Results of experiments on this question have been reported (17). The rapid outward movement of radioactive potassium from barley roots into salt solutions was not accelerated by increasing temperatures nor by higher oxygen concentrations in the bathing medium, but occurred even under anaerobic conditions. In this latter case, efflux by simple diffusion, as measured by the net passage of solute into distilled water, accounted for about one-half of the outward movement of potassium¹¹. It is true, however, that the potassium exchanges or efflux by simple diffusion, observed under the experimental conditions, were relatively small. Only about 10% of the total K present in the roots was subject to rapid isotopic exchange (57). Possibly constituents of the cell wall and external cytoplasmic surfaces were mainly involved.

The exchange adsorption of cations is not related in a simple manner to those metabolic factors which will be shown to be necessary for net ionic accumulations. It should be noted that this exchange of ions was distinguished from that more limited exchange which occurs in non-living tissues. The exchanges primarily studied in these experiments, on the contrary, were associated with the normal state of the living tissue and involved regions more deeply seated than the root surfaces.

On the basis of experiments on *Nitella*, using radioactive isotopes, three phases were suggested as operative in the net influx of ions (10, 11, 13-15, 52): first, a strictly limited exchange adsorption of ions (I^{\pm}) in the external medium for others previously

¹¹ The magnitude of the exchange under various oxygen concentrations is similar and appears to be confined to limited regions of the tissues, possibly cell surfaces. Under all gas conditions the rate of continued exchange is very low. Under anaerobic conditions or other treatments leading to a deficiency in aerobic metabolism the extended exchanges cannot be expeditiously studied. Here two factors are involved; first, the inherent rate observable is low even under favorable conditions; second, the continued penetration of solute is possibly impaired by a decreased permeability of the protoplasts of the tissue (36).

existent in the wall or cytoplasm of the cell, phase I; second, a recurring alternation of ion flux in the cytoplasm consisting of two phases, II and III. In phase II, a desorption of ions occurs, consisting of a displacement of adsorbed ions (I^{\pm}) from cytoplasmic salt linkages by ions produced in metabolism, this metabolism enhanced by the prior entry of the ions (I^{\pm}). In phase III the metabolically produced ions adsorbed on cytoplasmic colloids are considered to be replaced by ions from the external medium. Release of desorbed ions from the cytoplasmic complex during the recurring phase II are conceived as supplying ions which by simple diffusion may move either out of the cell or penetrate farther inward, or both; inward, finally to vacuolar storage regions. The primary exchange adsorptions of these investigators appears to be of the same nature as that suggested by Steward (70) as "induced", and in the present paper as non-vital exchange adsorption (after experiments of Broyer and Overstreet (17)). The net result of the alternating exchange phases II and III would appear to correspond with a vital exchange adsorption of solute. Brooks and Mullins suggested that the grand period of accumulation is related alone to this recurrent exchange mechanism (net of phases II and III). But, it should be noted that in the studies on roots there is no evidence of alternating ad- and desorption phases. Perhaps in these multicellular organisms there would exist overlapping phases, so that the periodicity would be masked.

The metabolically formed ions involved were suggested by Brooks and his collaborators to include organic cations and anions, hydrogen and bicarbonate. However, experiments of other investigators (17, 35, 70) and unpublished results from this laboratory are considered as suggestive of an accumulation process governed by a mechanism not related merely to exchanges for metabolically formed ions of the cell. Under certain cultural conditions, differences in hydrogen, bicarbonate and organic cation and anion concentration between the external medium and the plant would appear to be in accord with the mechanism suggested by Brooks and to a lesser extent with that of Lundegårdh (46, 47). However, assuming the single process of exchange adsorption as operative, carbon dioxide concentration differences would not appear to be adequate to explain the trend of net solute accumulation (58). Organic ions, although they appear in significant quantities in culture

solutions, can not suffice to quantitatively account for net cation or anion accumulation.

Stiles and Skelding (76) distinguish two phases in the net influx of salt into carrot tissue. The first phase is considered to be primarily associated with an adsorption exchange phenomenon. "The factors determining the second phase of absorption are at present obscure. They may include ionic exchange between external solution and internal cells of the tissue, permeability changes, respiration, and other metabolic activities".

Ready observation of ionic or molecular (including ion pairs) exchange of non-metabolites may depend on the presence of sufficient easily replaceable cation in the protoplasm, particularly associated with a "high salt" condition of the tissues. Under "low salt" situations exchanges may be less readily observable. Exchanges involving solute in the vacuole, or internal lumen phase, would be less readily accomplished than between the external medium and the cytoplasm. Exchange of both cations and anions would invoke the postulate of a mosaic protoplasmic structure (6-9, 15, 46). Results of Mason and Phillis (49) on cation-anion retention by tissue residues, after expression of essentially all available fluid, would suggest a favored situation for cations. The net charge on the surfaces of roots (46), as indicated by colloidal suspension studies, suggests a predominant number of available cation adsorption linkages. The complex association of protein "Zwitter-ions" (ampholytes) in the protoplasm is possibly involved (15, 18).

Under certain conditions it is possible that the mechanism for continued exchange adsorption alone may affect a large proportion of the net influx of solute, although the amount may not be of great magnitude. Maintained exchange adsorption of this type must be dependent on the outward movement of metabolically formed solute or continued increase in exchange capacity of living cell colloid. Hydrogen and bicarbonate ions, and possibly organic cations and anions, may be of importance in this former regard (compare 59). In general, surface phenomena can not account for the high concentration ratios of certain solute species between the internal and external solutions of the typical plant systems. Such accumulations could be attained only through a metabolic accumulation mechanism, although the other processes may be indirectly involved.

Metabolic Accumulation: Experimental data have been reported

(34) on the general nature of salt accumulation by abscised roots of barley, and reviewed by Hoagland (32, 33; also see 82). This process was shown to be related to metabolism and modified by factors influencing the latter. Temperature and oxygen supply were two of the major factors influencing the rate of metabolism and the accumulation of solute. These experiments and those of Steward and his associates (70) showed that accumulation is related to metabolic activities associated with the cytoplasm. This relation was indicated earlier by studies with *Nitella* cells (37, 39) and with intact barley plants (30). (Also compare studies on *Valonia*, reviewed in 1936 (54), and on storage tissues (76, 64, 65).) Brooks (10, 11, 15), using *Nitella*, has presented data, based on the use of radioactive isotopes, concerning relationships of concentration, for several inorganic cations, between the cytoplasm, the vacuole and the external medium. These relations, under the conditions of his experiments, suggested that a concentration difference between the large central vacuole and the cytoplasm may exist, at least in an initial phase of ion accumulation, higher in the region within which the metabolic accumulatory mechanism arises, namely, the living cytoplasm. The central vacuole under these conditions would act as a passive, simple diffusion reservoir. It remained to be determined whether or not an inverse relation, with the concentration of the solute species higher in the vacuole than in cytoplasm, could be attained under other conditions (compare Collander (23)). It was shown subsequently (36) that the concentration of bromide in the vacuoles of *Nitella* cells could become higher than in the plant residue composed of the cytoplasm and walls. A similar relationship was found between the fluids of the xylem, obtained as exudates from decapitated barley plants, and the composite expressed sap of the bleeding roots (36) (compare 47). Such a result might suggest that the movement of solute from the cytoplasm to the internal lumen was against the direction in which the concentration of the solute species decreases. However, attention may be called to the uncertainty which attaches to any interpretation of results on the cytoplasmic fluids capable of separation by available methods (16, compare 2, 3, 62). Solutes might be present, for example, within the cytoplasm in minute vacuoles or other aqueous phases, or more intimately associated with the protoplasm itself and thus subject to undefined forces of retention.

As has been noted (27), such a result may rather be related to non-solvent (bound) water effects (compare 49, 15). Some of the water of the cytoplasm may not function as a solvent for the solute species. It is evident, then, that the concentration of the constituent solute in the remaining water would be proportionately greater. If the latter explanation for the apparent concentration difference is correct, the true concentration difference may be even in the opposite direction.

Some investigators believe that the binding of water, if any, is ordinarily important only in tissue systems containing very little water (15, pp. 25-30; compare 18, pp. 233-239). With this viewpoint a resultant two-fold difference in concentration of a constituent solute in favor of the vacuole or internal lumen (36) could not be explained by invoking the principle of bound or non-solvent water *in vivo*.

The metabolic accumulation of a solute species, across the differentially permeable interposed cytoplasm, is a unidirectional (polarized) movement associated with conditions of oxidative, generally aerobic¹², catabolism. Accumulation of electrolyte by this process is characterized by an equivalent net influx of cation and anion. Although a high rate of metabolism seems to be generally observable under conditions of rapid salt accumulation, thermodynamically a very small amount of the total energy released

¹² Excised roots of aerobic plants normally adapted to culture environments relatively low in oxygen need to be studied in this regard. In these latter cases the fundamental requirements may be essentially the same, the intact plant in its normal habitat deriving a greater proportion of its oxygen from the aerial environment through specialized structures (19, 25), and in certain cases to a limited extent through symbiotic relationships with other organisms, as algae. However, the possibility also exists that for certain plant species, different conditions of aerobiosis may be beneficial or necessary for metabolic accumulation of solute. Although our rooted plants are generally of aerobic type and essentially autotrophic, anaerobic organisms exist in nature. These latter, and under certain conditions the intermediate forms, are particularly dependent for their ability to accumulate solute on intramolecular catabolism of compounds contained within the organism or derived from external sources. The supply of energy to heterotrophic organisms, aerobic or anaerobic, for net anabolism will depend upon the prior elaboration of oxidizable compounds by associated or foreign autotrophic organisms, which latter derive their internal energy ultimately through photosynthesis. It may be emphasized that in all cases the presence of transference compounds capable of ready oxidation and reduction, catalyzed by certain metals or enzymes, are undoubtedly involved (36, 46, 47). Some specific reactions, dependent on steps of aerobic respiration, or possibly in some organisms anaerobic respiration, characteristic of the organism, are directly associated with the metabolic energy transfer for accumulation of solute.

would be required to accomplish the accumulation of the solute by cells (81, compare 64). It may be pointed out that the required energy calculated in this manner is simply that necessary to accomplish the difference of concentration of a solute species between the external and internal phases of the system and is independent of any particular process by which it is effected. If we may assume that the continuous aqueous medium of root cells (through which heat transfers would probably be rapid) admits of thermal equilibrium within the system, any mechanism suggesting the utilization of thermal energy gradients for the accumulation of solute would be doubtfully involved (28; compare 47).

The over-all net accumulation of a solute species is a "selective" unidirectional (polarized) process whereby substances are moved across the interposed living differentially permeable cytoplasmic region. This flow is generally inward¹³, into vacuoles of cells and the lumen of the xylem (21, 29a). Metabolic accumulation of solute is characterized by a high temperature coefficient. The process is directly related to oxidative catabolism of the organism. The accumulation limits are defined by the concentrations of solute and food in the cytoplasm and the rate of oxidative catabolism. Metabolic solute flux is directly linked with metabolic solute free energy. This energy arises through an enhanced respiratory activity within the living cytoplasm. The increased rate of respiration is also characterized by a high temperature coefficient. The cytoplasmic, or protoplasmic, "pump" is visualized as a living machine whereby energy from oxidative catabolism can be applied, within the protoplasm, to raise the free energy of the particles of a substance such that they may tend to flow with and against the direction in which their concentration decreases between two solution phases separated by the differentially permeable membrane. (Compare the definition of a physico-chemical membrane, by Sollner (67a).) The initial step appears to be a cytoplasmic, or protoplasmic, catalytic interaction between the cytoplasm (membrane) and the solute whereby an interconnected release of oxidative catabolic energy and

¹³ Whether some experimental data with fungi indicate that the polarity may be directed outward in certain cases, for example, in the efflux of oxalic acid, remains to be studied. Compare the movement of solute through the integrated osmometer systems of the animal. In certain animal organs a migration, against the direction in which the concentration of the solute species decreases, is unidirectionally from the blood to a lumen; in others, from a lumen to the blood.

influx of solute is initiated (36, 47, 64). The cytoplasm is relatively impermeable to solute at all times (in the direction perpendicular to the osmometer membrane surface), at least in the endoplasm or at the vacuolar surface, as long as its organization is not too greatly impaired. The permeability may, however, vary in both space and time, and with the type of solute to which the cytoplasm is exposed (5, 11, 15, 22, 54, 55, 56). Increase of the solute free energy difference between the external and internal phases of the plant systems, nevertheless, tends to move solute species across the interposed cytoplasm. One concept of solute flow involves the movement of a solute species, as molecules, ion pairs or ions, into the vacuole, for example, although membrane properties prevent them from readily moving outward. Another concept is that of a dynamic system through which solutes are metabolically transported to the internal phase (as a net effect) and the substances moving outward are retransported inward, except as certain simple diffusions and exchange adsorptions take place (36).

It should be emphasized that in any living system in which equation 9 or 14 applies, the movement of a solute species against the direction in which its concentration decreases, apart from those migration influences which may tend to cause a lowering of the specific free energy of the solutes, requires the expenditure of metabolic energy. This applies, for a non-electrolyte, to the solute molecules; for an electrolyte, to the molecules or its constituent ions, both anion and cation. The enhanced oxidative catabolism, related to the accumulation of a solute, is effective in tending to cause both cations and anions, or ion pairs or molecules (at least as a net result) to move toward equality of escaping tendency or free energy. Since cytoplasmic permeability is not constant with time, a variable part of the oxidative metabolic energy may be expended in overcoming resistance to flow (included in the permeability coefficient), manifested through a chemical to thermal conversion, and therefore a stoichiometric balance between the solute accumulated and the enhanced catabolic energy related thereto may not be realizable in all experimental results.

RELATED ASPECTS OF SOLUTE FLUX

Metabolic Accumulation and Enhanced Oxidative Metabolism:
The enhanced oxidative catabolism associated with the net influx

of solute have been related in various ways by investigators (see footnote 7). Lundegårdh (45-47) considers the increase in respiration, estimated primarily from CO_2 evolved, to be related directly to the influx of the anions of the salt entering the system. The associated cation may modify the "anion" respiration, but is not the governing factor in the intensity of the increased respiration. The anionic effect is believed to be dependent upon the predominantly negative electrochemical mosaic nature of the interface between the cytoplasm and the external medium. The "anion" respiration is viewed to be required to overcome the repulsion, and allow an interaction to take place, between the anion and the cytoplasmic surface. A straight line function between the total respiration (CO_2 evolved) and anion influx is assured. Extrapolation of these curves to zero influx made possible the estimation of values for "fundamental" and "anion" respirations.

The change in oxidative respiration associated with the process of solute accumulation has been stressed by others. Steward and collaborators have observed an increase of respiration (CO_2 evolved) in monovalent salt solutions and a decrease in divalent salt solutions, using excised discs of potato tissue. They visualize an increase of respiration attending salt accumulation, but not necessarily in stoichiometric proportion. In these tissues, classed as dormant matured cells capable of renewed accumulation of solute through restoration to active growth and metabolism (69, 70), concomitant increase in protein content of the system is required. The biochemistry of this process has been extensively studied (72).

Robertson, using excised discs of carrot tissue, has found an increased respiration (O_2 involved or CO_2 evolved) attending salt accumulation, which is termed "salt" respiration in contrast to a "ground" fraction observed when the tissue is placed into water (64, 65; compare 46, 47). The "salt" respiration, when plotted against the salt accumulated, results in a positive exponential function, *i.e.*, as the amount of salt accumulated is increased, the "salt" respiration per unit salt accumulated is increased, as required thermodynamically. The respiratory response of carrot tissue is similar to that of potato when placed into solutions of either mono- or divalent salts, varying in point of time. A high temperature coefficient for both salt accumulation and "salt" respiration was shown. Adequate aeration was required as with other types of tissues (34, 47, 70).

Experiments with abscised barley roots afford evidence which also leads to the conclusion that the net accumulation of salt by living cells is directly related to aerobic catabolism (27, 28a, 29, 29a, 31-34, 36, 48, 78, 80). Net salt accumulation is accompanied by an increased total respiration (O_2 involved or CO_2 evolved). Here, the net influx of all types of salts studied was associated with an increase in respiration. Accumulation of salt is arrested under anaerobic conditions. Increase of temperature is accompanied by an increased rate and equilibrium values for salt accumulation. Although a high temperature coefficient is indicated, accumulation can proceed at low temperatures under aerobic conditions. Mere CO_2 evolution is not necessarily sufficient for the net influx of solute (see also 47). Thus, accumulation will not occur at anaerobic oxidative catabolic levels above those allowed by low temperature aerobic conditions (36). Certain requisite steps, here aerobic, of oxidative catabolism are indicated as directly related to the process of net salt accumulation¹⁴.

Influence of Respiratory Regulators: Because of the importance of aerobic respiration in relation to salt accumulation, it was an obvious step to investigate the influence on net salt influx, of substances which could alter the course of respiration. The influence of a hydrogen acceptor, methylene blue, was studied from this viewpoint. The presence of this substance in the external medium did not increase accumulation. Higher concentrations of methylene blue inhibited accumulation without depressing the carbon dioxide production. Permeability of the cells was increased, indicated by the net outward movement of solutes, but disturbance of the protoplasmic mechanism prevented salt accumulation (36).

Under anaerobiosis either or both of two factors may be influential in modifying the course of solute accumulation. Firstly, there is the possibility of failure of synthesis of some requisite protoplasmically active compound. Results from application of respiratory inhibitors, to be discussed hereinafter, suggest this

¹⁴ The discussion has been limited primarily to the accumulation of electrolytes. Evidence is wanting on the net influx of apolar compounds. Many cases exist in plants wherein non-electrolytes occur in concentrations higher within the plant cell than without. These solutes are normally not readily lost from the cell or organ, but are retained in a manner similar to that for electrolytes. This situation would suggest that if conditions were favorable, such substances could likewise be accumulated by the cell from its external bathing medium. One unpublished result indicates that compounds of low dissociation in water (for example, xylose) may take place.

probable influence. Secondly, formation of a deleterious product of metabolism might also inhibit the accumulation of salt. The influence of oxygen deficiency in preventing salt accumulation might, for example, be associated with the formation of by-products of fermentative processes. Experimental application of alcohol or lactic acid in the medium bathing abscised barley roots did not significantly modify the course of salt accumulation (36).

Early results of Lundegårdh with decapitated wheat seedlings (46) showed that the influx of salt into the tissues was inhibited by the application of cyanide in the bathing medium. The respiration correlated with the anion influx by control sets of roots was completely prevented, while the "fundamental" fraction was unmodified. Similar conclusions have been drawn in recent studies using discs of carrot tissue (65). Some of the data in the latter studies would suggest that both the "salt" and "ground" components of respiration were affected, at least by high concentrations of cyanide. Experiments with abscised barley roots showed that salt accumulation and the normally concurrently increased respiration were both inhibited by low concentrations of cyanide (36). Higher concentrations of cyanide reduced the total values of carbon dioxide evolved to amounts considerably lower than those produced by roots in distilled water. The general effect was likened to an induced anaerobiosis. The general suggestion was that a metal-catalysed respiratory system was linked in some way with the process of salt accumulation, although the exact nature of the system has not been elucidated (33, 36, 46, 47). The above quoted results with barley roots have been confirmed and extended (48). In these latter experiments, the effect of cyanide, azide, iodoacetate and malonate on salt accumulation and concurrent respiration (O_2 involution and CO_2 evolution) were studied individually. Cyanide and azide were found to inhibit approximately two thirds of the respiration, and completely prevented bromide accumulation. Cytochrome oxidase was suggested as the enzyme affected. Partial cyanide inhibition caused a disproportionately large inhibition of salt accumulation. This seemed to suggest a second unknown action of cyanide more directly related to salt accumulation than cytochrome oxidase inhibition. Iodoacetate and malonate inhibited both net salt influx and oxidative respiration. Malate, succinate, fumarate and citrate reversed the iodoacetate inhibition of both respiration and salt ac-

accumulation. They also counteracted the malonate inhibition of accumulation, but had no significant effect on the respiratory values. It was suggested that a cycle similar to the Szent-Gyorgyi dicarboxylic acid cycle or the more extended citric acid cycle of Krebs was an integral part of the respiratory system interrelated with salt accumulation.

Miscellaneous Ionic Effects: Simple interionic effects have been recognized to occur in the influx of salt into plant cells. In dilute concentrations in the bathing medium one ion may modify the rate of entry of another species. These effects are involved between cations, between anions, and between anions and cations (24, 25, 30, 38; compare 68). When a deleterious result on a living cell or organ, caused by the presence of a high concentration of a single salt in the bathing medium, apart from any secondary effects due to adverse water relations, is ameliorated by the simultaneous application of a second solute species, even in relatively minute concentration, this action is termed "antagonism" (1, 29, 54, 73). The neutralization or modification of solute flow in these cases may be related in part to mere mutual exclusion from association with the effective protoplasmic surface. The effect may be more intimately related to the respiratory mechanism or the constitution of the cytoplasm through which the solute must pass. The latter possibility is brought forward more clearly through studies of Viets (80). It was found that the influx of a single monovalent salt species, and concurrent increase in aerobic respiration, could be increased by the presence of any one of several divalent or trivalent cations in the external medium. The primary effect here may be either on the membrane or on a surface metabolism intimately related to the permeability of the cytoplasm. The possible surface effect "due either to the presence of the polyvalent cation or to its penetration into the surface might be conceivably an effect on some phase of metabolism concerned with permeability, as dissociation of membrane components, configuration of protein chains, hydration of colloids, etc. This surface effect would demand that during rapid accumulation the resistance to ion movement offered by the protoplasmic surface is a link in relative minimum in the chain of links involved in the complex mechanism of salt accumulation from single salt solutions. Under anaerobiosis some other link in the chain becomes limiting and no effect of polyvalent cations is apparent".

Solute "Selectivity" in Plants: The differential permeability of the membrane of the reference osmometer systems of the plant to solute has been observed for many years. Extensive studies have been made of these relations (20, 21, 24). Striking comparative examples are presented by *Valonia* and *Halicystis* (compare also *Nitella*; 54, pp. 295, 296), related unicellular algae which both normally inhabit sea water. While for certain ions simple diffusion equilibria do not appear to be attained, other ion species are differentially accumulated within the vacuolar solution. The complete nature of the differential permeability of the cytoplasmic membrane as a whole or its limiting surfaces is still to be elucidated. This "selectivity" for net influx of solute certainly involves the self-regulatory activity of the living cytoplasm, *i.e.*, the protoplasmic colloid pattern fundamentally governed by the metabolism characteristic of the species of organism under the environmental conditions imposed.

ON THE RATE OF MOVEMENT OF MATERIALS INTO PLANTS

In the plant as a whole, substances will tend to move independently, each constituent solute component or water tending to flow with the direction in which its specific free energy decreases. In any case, the tendency for movement at any instant will be completely expressed in terms of the net specific free energy tending to cause a constituent of a component in solution to move through or across a limiting osmometer membrane, namely, the net influx specific free energy (NIF). The rate of net movement of a constituent of a component in solution is proportional to the net influx specific free energy gradient (NIFG), where the coefficient of proportionality is the permeability of the membrane (k_p). In other words, the rate of net influx is equal to the product of the permeability of the membrane and the gradient of the net influx specific free energy. Here, this gradient is the measure of the space-rate of change of the net influx specific free energy with dimensions of $\text{mL}^{-1}\text{-t}^{-2}\text{L}^{-1}$. The permeability, a property of the membrane, is defined to be equal to the mass of the constituent component which moves, in unit time, through a unit area of membrane under a unit gradient of net specific free energy. Since the energetic tendency for movement of materials through or across a membrane is a process of diffusion, the direct analogy between the coefficient of simple diffusion and the coefficient of permeability is obvious (15).

A dimensional analysis of the permeability of the membrane and the rate of net influx of a constituent solute species or water, in a solution, may be outlined as follows (compare 15, pp. 8-10): "Estimation of the permeability of a membrane": The permeability coefficient (k_p) is defined by the dimensional relation $k_p = \frac{m}{t} \times \frac{1}{L^2}$

$\times \frac{1}{L} = t$. "Estimation of the rate of net influx" of a constituent

solute or water: The rate of net influx (RNI) equals $k_p \times \text{NIFG}$

or $\text{RNI} = t \times \frac{m}{L^2} \times \frac{1}{L} = \frac{m}{L^2 t} = mL^{-2}t^{-1}$. In words, the rate of net influx is measured by the mass of a constituent, of a component in solution, which moves through a unit area of membrane in a unit time.

Since the thickness of the cytoplasmic or protoplasmic membrane of a particular osmometer is not known with any accuracy, yet assumed to be constant during time t , the permeability coefficient and the net influx rate may be each expressed more practically by omission of this factor (membrane thickness) from both of these expressions. The dimensions of permeability are then tL^{-1} ; those of rate of net influx are the same as before, *viz.*, $mL^{-2}t^{-1}$ (see 15a, p. 28 on rate of exudation).

INTERRELATION BETWEEN THE MOVEMENTS OF WATER AND SOLUTE

Although the movements of water and a constituent solute have been discussed in separate disquisitions (for simplicity and clarity) and the net tendencies toward flux are independent physico-chemically, the movement of one component will modify, directly or indirectly, the subsequent flow of the other component. For example, accumulation of solute internally through metabolic accumulation will lower the specific free energy of the water molecules internally, leading to an inwardly directed osmotic "solute" specific free energy. Similarly, influx of water, to satisfy a difference in osmotic specific free energy, will modify the solute specific free energy, and the hydrostatic specific free energy relations of both the solute and water components of the system. Thus, the movements of water and solute into plants are interrelated, though independent energetically. The complexity of the resultant movement

of materials into plants is indicated. At any time t , the concentration of a constituent solute or water component in a solution within the plant is the net result of all the possible modes of movement involved during the previous period of time. In other words, the time course of the net flux of a constituent component is the integrated resultant of all of the possible modes of movement of both components of the system involved during the interval.

SUMMARY

The movement of solutes into plants can and should be expressed in terms of the specific free energies of the solute molecules. Similar expressions have been developed for the flow of water. The mathematical relation between the specific free energies of the solute and the water in solutions and in the osmometer systems of the plant has been presented. The terminology is consistent with thermodynamic and physico-chemical principles.

The movement of solutes into plants can be accomplished through several modes of action. These possible modes of flow include simple diffusion, Donnan equilibria, pressure effects, adsorption including exchange adsorption, and metabolic accumulation.

The metabolic accumulation of solute is probably a universal mode of net influx. The accumulation is directly related to oxidative catabolism for its source of energy. This energy is applied to the solute molecules through some type of solute-cytoplasmic interaction increasing their free energy such that the molecules of a solute species tend to move with and against the direction in which their concentration in solution decreases. The mechanism for conversion or transfer of chemical into mechanical energy remains to be elucidated.

SUPPLEMENTARY STATEMENTS

Diffusion, using the term as defined in parts I and II, and by others (44), includes in addition to concentration or activity effects, consequences of all other influences which may modify the free energy of matter within a system. If this definition is adopted generally, the term *simple diffusion* can be used for the restricted effects of concentration or activity differences of a constituent component in solution within a system. However, for the scheme proposed here, it may be preferable to limit the term diffusion to its

earlier usage. The following terminology is then recommended. *Flux Defined*:—"Flux" is the spontaneous movement of matter, as a flow or migration in response to a difference of free energy, working towards equilibrium of escaping tendencies. With this definition of flux, a more restricted way of using the terms *flow* and *migration* is desirable. Thus, *flow* may be used where the flux is predominantly a mass movement of material relative to a conduit; *migration* where the course is primarily the result of the mean motion of the particles of one constituent component, relative to those of another. *Diffusion Defined*:—"Diffusion" is the process of flux (a migration) where restricted to a response to a difference of concentration or activity of a constituent component in solution within a system.

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THE ESSENTIAL NATURE OF CERTAIN MINOR ELEMENTS FOR PLANT NUTRITION. II¹

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INTRODUCTION

Work on the relations between "minor" or "trace" elements and plant growth continues to proceed rapidly, and the literature on the subject becomes correspondingly voluminous. During the recent war years the necessity for increasing food production has led to attention being concentrated on the possibilities of raising crop yields by the use of appropriate minor elements in addition to the usual fertilisers. At the same time the harmful effect of excess quantities has not been overlooked. During this period little further progress has been made on the fundamental question as to whether various minor elements are essential for growth, using the word "essential" in the strict sense that in the absence of the element in question growth is inhibited. The present survey of necessity covers a wide field, and it is possible to indicate only the general lines along which advance has been made since the two earlier reviews were published (29, 30) with references to a selection of papers which illustrate the points described. Willis's bibliography (160) with six supplements gives a very comprehensive collection of abstracts on the subject, and for other collected references on the mineral nutrition of plants papers by Arnon (8) and Sommer (138) should be consulted.

ARSENIC

With arsenic attention usually focuses on its poisonous properties rather than its problematical beneficial character in minute quantities. Normal plants may contain appreciable amounts of arsenic, absorbed and stored without apparent injury. Some seaweeds contain, when fresh, as much as 20 p.p.m. (on the dry

¹ Supplement to article in *The Botanical Review* 2: 173-196. 1936.

weight) of arsenic trioxide, but this is reduced to 2-4 p.p.m. on bleaching (2). Sudan grass (*Sorghum vulgare* var. *sudanese*) and bush bean (*Phaseolus vulgaris* var. *humilis*), when grown with non-toxic strengths of sodium arsenite in the nutrient solution, may store up arsenic in their tops which is in excess of the limit established by law for the arsenic content of foods (97). Sudan grass is more tolerant than bush bean to arsenic in the nutrient solution as it takes 12 p.p.m. arsenic to kill it, against only 1.2 p.p.m. needed for bush bean. The yield of tomatoes may be markedly reduced by as little as 0.5 part As per million (39). With increase in available phosphorus the plants were found to tolerate greater concentrations and to absorb less arsenic.

Use of arsenical sprays in orchards raises the important issue of the possible harmful effect on other crops. After repeated sprayings sufficient arsenic may accumulate to be toxic to such crops as alfalfa and barley (80). Treatment of the soil with three to five tons of ferrous sulphate (copperas) per acre reduced the amount of soluble arsenic and increased fertility. Without treatment soils may gradually fix arsenic so that successive crops show less arsenic injury (164), the toxicity also being reduced by increases in the organic matter of the soil. Arsenical weed killers used to clear off old crops, such as cranberry, were found to affect new cuttings adversely when the land was replanted (48). As arsenic was noted to be held for several inches down, deep ploughing was advocated to put the treated soil out of reach of the new cuttings. Peach trees are also very sensitive to arsenic absorbed from the soil, as signs of toxicity appear if amounts greater than 2 p.p.m. occur in the dry material of the leaves (93).

One instance of stimulation by arsenic shows that growth of oat seedlings is improved by up to 0.25 p.p.m., beyond which poisoning occurs, toxicity increasing with the concentration (158).

BORON

Boron easily retains first place among the trace elements in the amount of observational and fundamental research carried on with regard to its relations with plants. In spite of war conditions scores of papers have appeared during the last few years, many indicating increased interest in the part played by boron in plant economy.

Improved tests for boron in soils and plant materials have been elaborated, the quinalizarin color reaction giving satisfactory results (17). The range of available boron in soils is considerable, and distribution of boron in soil depths varies from place to place. In typical Hawaiian soils twice as much boron was present in every case in the surface soil as in the corresponding subsoil (43), whereas in Florida there was little difference between the boron content of the top- and subsoil, whether under virgin or cultivated conditions (4). It has been suggested that the plant may be a better indicator than the chemist of available boron in the soil. Availability of boron was more clearly shown by tissue analysis of stems and leaves of sunflowers than by analyses of the soil on which they were grown (46). With plants grown in limited amounts of test soils the criterion of the boron status of the soil is the age at which deficiency symptoms appear (40). In oranges grown in Florida the boron content was higher in the plant ash than in the soils (36). In tomatoes the concentration in leaflets was significantly increased when the supply was stepped up in the sub-stratum. Water culture experiments indicate that this correlation between the amount of boron absorbed by the plant and the concentration in the substratum is irrespective of the boron compound present (41). The demand for boron increases with heavier doses of fertilisers (135), fast growing plants responding more to boron additions than do slow growing species. Boron may be present in the soil in mineral and organic forms, the former being leached out in acid soils. The organic compounds split up, and if lime is applied the boron may become fixed in stable and unavailable forms (81). The fertilising properties of alluvial soils are partly attributed to the boron they contain (86).

The percentage and total quantity of boron contained in plants varies not only with the species but with the soil, manuring, rainfall, liming and other environmental factors. Spring wheat from 18 Russian provinces varied in boron content from 3.7 to 10.2 mg. per kilogram dry matter (25). French work showed that *Taraxacum vulgare* from two fields contained 14.1 and 80 mg. per kg., respectively (23). Monocotyledons contain less boron than dicotyledons (131), members of the Papilionaceae and Cruciferae being specially rich in it. Analyses of various agricultural plants showed a more limited range of boron content in the seeds than in

the entire plant (24). Boron analyses have been presented by various workers (15, 111) showing the effect of liming (104) and of the time of sampling on the boron content (124).

Indications of an association between calcium and boron in the metabolism of plants were recognised in the earlier days of boron investigations (157), and recent work has confirmed this. Excess lime inhibits the uptake and the utilisation of boron (122). Boron starvation results when the balance between calcium and boron become unfavourable, the necessary ratio for healthy growth varying with different species (49). Experiments indicate that the ideal balance for tobacco is about 1,200 of calcium to 1 of boron, for soy beans 500:1, and for sugar beet about 100:1. If this balance is upset by a low intake of calcium, as occurs on acid soils, very little boron will cause injury to the plants. On the other hand, where too much calcium is available, as on overlimed or alkaline soils, boron starvation may result, owing to deficiency of available boron (79).

A relationship between boron and calcium nutrition has also been deduced on anatomical grounds. In boron-starved leaves of cabbage the cambium and phloem are replaced by undifferentiated parenchyma. Addition of boron to the nutrient solution or immersion of boron-deficient leaves into solutions of boron or calcium salts stimulates the differentiation of this parenchyma into wood vessels, indicating a close relationship between the two elements. (87).

Potassium also has a bearing on boron deficiency and toxicity. At low levels of available boron, deficiency symptoms in tomatoes and maize are accentuated by increasing doses of potassium (119). At high levels, on the contrary, boron toxicity increases as the potassium level rises. In this respect calcium and potassium act in opposite directions. Reeve and Shive (120) state that potassium appears to influence the response of the plant to boron indirectly through its determinative effects upon the processes involved in the absorption and accumulation of calcium.

The interaction between boron and plant growth is much influenced by environmental conditions. It was already known that in *Vicia faba* and certain other plants boron deficiency symptoms are less pronounced under short-day than under full-day conditions (156), and this is specially marked in radish (132). It has been

claimed that the presence of boron in the soil tends to reduce growth at lower temperatures and to diminish resistance to cold (125). The degree to which different parts of the same plant can resist cold varies according to the mineral content of the parts concerned. Photosynthesis is affected if boron compounds are injected or sprayed on plants. Spraying *Vicia faba* leaves with a weak solution of boric acid resulted in a temporary lowering of the assimilation rate, followed by a marked increase (121). Spraying with small amounts of borax has been found as effective in controlling boron deficiency in sugar beet as application of larger amounts to the soil (33). Injection of boric acid was found to cause a reduction in photosynthesis, though this was attributed to too strong a concentration of the injected solution (152).

Much of the literature on the subject deals with the beneficial or harmful action of boron on various crops, frequently indicating parallel results from different parts of the world. Only outstanding points can be indicated here, but many of the papers are indexed in various summaries to which attention has been drawn.

Repeated confirmation has been obtained of the efficiency of boron in controlling or preventing heart rot in sugar beet. Though Chilean nitrate failed to exert a definite curative action on heart rot, it proved to be a more suitable fertiliser than synthetic sodium nitrate, containing no boron (108). In the production of sugar beet seed in Oregon, application of 25 to 35 pounds of borax in the early spring of the second year's growth effectively checked the appearance of boron deficiency symptoms on the seedstalks (149). Drilling of fertilisers containing borax in contact with the seed is apt to retard germination and injure the stand, while control of heart rot is less effective than when the borax is applied as a side dressing (101). Prolonged summer droughts lead to shrivelling of the root tips of sugar beet, and if the boron supply is deficient the beets are predisposed to bacterial gummosis, due to attack by various forms of bacteria, primarily *Bacillus betae*. On such infected soils at least three years should elapse before beet reappears in the rotation (126).

Blackening and internal breakdown in table beet is of great economic importance, and larger dressings of borax are needed for its control than for heart rot in sugar beet (116, 154). Histological changes in beets caused by boron deficiency largely affect cell di-

vision and differentiation (78), the form of the ensuing necrosis being similar in plants grown under widely different environmental conditions. The interaction between boron and certain major nutrients varies with the elements concerned, boron increments proving more efficient in increasing growth at the lower levels of calcium nutrition than at the higher, whereas with potassium the reverse occurs. It has also been suggested that the effect of boron deficiency may be to prevent formation of certain compounds, such as protopectins, rather than to destroy compounds already formed (94).

Confirmation has been obtained of the control of raan in turnips by boron, as little as 0.0681 p.p.m. of boron being sufficient for production of normal plants (161). Boron treatment of carrots and turnips on clay soil has given highly significant increases in storage quality (70). With deficient boron carrots are small and immature (163), but the effect of boron on increasing the carotene content is variable and irregular, even on the same soil in different seasons (71).

In potatoes boron deficiency may cause leaf roll resulting in a necrosis which affects tissues other than the phloem (130), but there is also evidence that small amounts of boron added to fertilisers may in some circumstances and seasons be toxic to potatoes and reduce the yield (72).

Throughout the United States much attention has been given to the value of boron treatment for improving the growth and yield of alfalfa. Emphasis is laid on the two types of boron deficiency, primary, due to actual scarcity of boron in the soil, and induced, caused by boron fixation brought about by liming (51). Plants showing severe boron deficiency symptoms were found to contain more boron in the upper than in the lower parts of the plant, the reverse occurring in plants with a normal boron supply (148). Seed production in various leguminous crops is also increased by an adequate boron supply (68, 112). While the function of boron in plants is still obscure, analyses show that the soluble nitrogen and carbohydrate content of alfalfa is affected by boron deficiency, and it has been suggested that boron may be involved in protein metabolism (129). With soy beans increased supplies of boron at mid-season have little effect on the boron content of the plant tissues,

whereas similar treatment either at earlier or later stages of growth gave a boron content which increased with the rate of application (74).

Boron deficiency is common in vegetables belonging to the genus *Brassica*, and Chandler has made an intensive examination of the various crops. Besides detailing the influence of boron on the growth, yield and root development, Chandler investigated the anatomy, showing that if boron-deficient plants are damaged the cork cambium is either abnormal, or, in severe cases, is not produced at all (37). All the symptoms of boron deficiency are regarded as secondary expressions of the alteration and retardation of meristematic activity, thus corroborating the views of earlier investigators. He does not, however, agree that simple chemical fixation of boron is brought about by the calcium ion, but suggests that the interaction of many other factors is probably involved (38). Cabbages, cauliflower and celery have been investigated by various other workers (44, 88, 153, 162).

The literature on the relation of boron to fruit crops continues to extend, but most of it simply provides additional evidence from various districts or different varieties. Heavy applications of borax to apple trees have been found to damage the keeping properties of the fruit (159). Abnormal growth and fruiting of grape vines is caused by boron deficiency, some varieties producing seedless fruits under such conditions (128). In pears boron deficiency causes symptoms similar to stony pit, a virus disease, but the latter is not corrected by boron applications (84). Cantaloupe, squash, tomatoes, peanuts, olives, jute, coffee, citrus and raspberries have all received attention of recent years in connection with boron nutrition, but space forbids even the mere listing of the work.

Another aspect of boron deficiency deals with its effect on the production of flowers, either for sale or for processing for various purposes. Nasturtium (34), gloxinia (7) and poppies (18, 28) all require boron for optimum flower production, and doubtless the necessity exists with many other cultivated species.

The boron literature is so voluminous and so much has perforce had to be omitted from this review that references to various bibliographies are appended for the benefit of those specially interested in the subject (10, 30, 31, 45, 155, 160).

COPPER

Evidence on the value of copper for plant growth is gradually accumulating, but most of the information is obtained in the field and comparatively little is based on fundamental work carried on under controlled conditions.

The striking work inaugurated by Allison, Bryan and Hunter (3) on the Everglades peat soil has been continued, and recent greenhouse experiments have indicated the necessity of copper for various plants (58). Piper, in Australia, studied different species in culture solutions with varying rates of copper, and described characteristic symptoms due to absence of the element (113). Analyses of crops from various districts indicated a greater variability in the amount of copper taken up by certain species on any one soil than in that absorbed by the same species on different soils (114).

The copper status on any area seems to be satisfactorily indicated by analyses of mixed herbage collected during the growing period. Where copper deficiency symptoms occurred in grazing stock the samples of herbage contained under 3 p.p.m. of copper on the dry matter, whereas 5 p.p.m. and upwards were found on healthy areas (13, 16).

The importance of copper in the prevention of reclamation disease is emphasized in a lengthy paper by Steinbjerg (142). The amount available to the plant seems to be at a minimum at pH 5.5-6.5, and the amount present in plants grown at this pH level is lower than that in crops grown on soils of higher or lower pH. The copper content of various plants varies widely, and this should be taken into account in arranging rotations. Copper deficiency can be compensated in various ways, as by the use of coke ash, compost, farmyard manure and also by copper sulphide and finely pulverised metallic copper, but exhaustive field tests are needed to clarify this phase of the problem.

Definite copper deficiency has been observed in tung trees in Florida, with abnormal and chlorotic leaf growth, defoliation and die-back of the shoots. Treatment with a variety of minor elements all failed, except with copper (50).

Widespread use of Bordeaux mixture as a spray led to consideration of its physiological action on plants. Photosynthesis of apple leaves suffered at least temporary reduction, regardless of the con-

ditions of temperature, light, humidity or soil moisture. The soluble copper fraction appears to be directly related to the retarding action. On the other hand, transpiration may be quite unaffected by Bordeaux mixture, though some depression may occur (139).

Treatment with minor elements induces enzyme responses which show considerable uniformity. This may indicate that these enzyme activities are expressions of general metabolic conditions within the plants rather than of direct influence of the minor elements. On the other hand, exceptional enzyme responses may be due to direct effects of a particular element, as the continued increase of oxidase activity with increasing addition of copper (11).

An interesting case of extreme tolerance to acidity and high concentrations of copper was observed in two fungi, *Acontium velatum* Morgan, and one of the Dematiaceae. Both grew in a nutrient medium saturated with copper at pH 2.0 to 0.3, and even made some growth at pH 0. It is suggested that this is the highest tolerance to acid and copper sulphate yet recorded for any living organism (140).

MANGANESE

The essential function of manganese as a plant nutrient is fully accepted, and much of the current literature provides additional evidence of the occurrence of manganese deficiency in various species and the value of treatment with manganese salts, either as fertilisers or as sprays. Manganese is also closely correlated with animal metabolism, and the question of manganese-deficient diets receives much consideration.

In a survey dealing with boron, iron and manganese, Shive (131) issues a much needed warning against the growing tendency to isolate the functions of individual trace elements: "While it is at present impossible to assign any one particular process as the special function of a given trace element it is probably safe to assume that each of these elements is a critical factor in every important physiological process involved in the nutrition of a plant".

Work continues in many areas on grey speck disease of oats, and repeated confirmation has been obtained of the value of spraying with manganese sulphate solution. Pot experiments indicate that in some cases the disease may be prevented by adequate moisture in the soil and be intensified by drought (60, 61). Spraying has proved effective in controlling the leaf symptoms of marsh spot on

various species of beans. Curiously enough, dwarf and haricot beans, which show the most marked leaf symptoms, are the most resistant and may remain free from marsh spot in the seeds, even when the leaf symptoms are very severe (73). On some soils the cause of manganese deficiency appears to be biological, due to an excess of micro-organisms which actively convert manganese sulphate to the oxide form, thus reducing the available manganese below the minimum level required for the normal development of oats (99). In Illinois the manganese content of several fully grown grasses ranged from 32 to 450 p.p.m., the manganese uptake being increased by phosphatic fertilisers (134) as well as by changes in the H-ion concentration. Similar results have been obtained with the leguminous forage crops *Melilotus alba* and *Lespedeza stipulacea* (1).

Various aspects of the relations of manganese within the plant have been considered. In tomatoes the chloroplasts are the first to be affected by a deficiency. Some of the food stored in deficient leaves is in the form of fat, but though that in the stems is starch the grains are mostly simple and few in number, in contrast with the compound and numerous grains in healthy stems (55). Chlorotic fronds of bracken suffering from manganese deficiency contained less starch and only 1/27 as much manganese as normally developed fronds (76). One function of manganese is to catalyse the reduction of nitrates to amino-compounds, and manganese-deficient plants therefore tend to exhibit high concentrations of nitrate (90).

The iron-manganese ratio is of considerable importance in plant metabolism. In soy beans symptoms of iron toxicity correspond to those of manganese deficiency and *vice-versa*. Iron and manganese are definitely inter-related in their metabolic functions, the biological effectiveness of the one being determined by the proportionate presence of the other (137). It also appears that while the iron-manganese ratio is of such importance in the plant metabolism, the absolute concentration of those elements that are available within reasonable limits does not seem to be of great significance (136).

MERCURY

Recent work with mercury has largely been correlated with its use as an agent to control diseases caused by fungi and bacteria,

with enquiries as to the resulting effect, if any, upon the growth of the crop plant itself. Organic mercurial disinfectant used for control of seed-borne diseases of wheat tended to retard the rate of germination, but increased the final percentage of seedlings (66). In chrysanthemums, Grace (65) found that rooting of chrysanthemum cuttings was increased by about 5% with organic mercury treatment.

With flax Muskett and Colhoun (105) state that suitable organo-mercury preparations gave good control of seedling blight, stem break and browning, the fibre yield, crop yield, or both, usually being increased. The increase of fibre may not be directly attributable to the control of seed-borne diseases, but may be due to a general beneficial effect due to seed disinfection. In contrast, Robertson (123) reported an apparent stimulation of the growth of mould on leather treated with low concentrations of 1 part in 20 or 40 thousand of an organic mercurial. Mercuric chloride, at about 1:1000 strength, has been much used against various corn-borne diseases of gladiolus and also for the control of thrips. Nelson and Cassil (107) investigated the relative absorption of the chemical by the unpeeled corms under different conditions of concentration, temperature and time of soaking.

Mercury cannot be used for sterilising grass seeds, as all compounds containing it inhibit germination (144). Seed storage in mercury vapour for six months had no adverse effect on the percentage of germination or in early seedling growth. Gray and Fuller (67) found there was a slight delay, but no reduction in percentage if germination took place in the presence of mercury vapour. Any mercury in the substratum hindered early growth, however, giving stunted plants, yellowing of leaves, failure of leaf development and early leaf fall.

Bacterial ring rot in potatoes has been effectively controlled and the crop yield increased by soaking the tubers in mercuric chloride at the rate of four ounces per 15 gallons, whole seed needing 20 to 30 minutes, but cut pieces only about 10 minutes (141, 52). Club root of *Brassica* also yields to mercury treatment, calomel (HgCl) being safer to use than corrosive sublimate (HgCl_2), the control in both cases being practically useful, though not complete (115).

Boer (27) has found that mercury compounds applied to the soil decompose with the production of metallic mercury, which is

eventually converted and rendered innocuous by formation of mercuric sulphide. Consequently the effects of mercury on plant life are not permanent, though they may last for considerable time, and mercury is thus not a cumulative soil poison.

MOLYBDENUM

During the last few years little has been published on the possibility of molybdenum being essential for plant growth. Nevertheless, evidence has accumulated from various parts of the world that a small supply of molybdenum is beneficial to the growth of certain crops, some soils being definitely molybdenum-deficient and on that account not carrying a full crop without special treatment. The molybdenum content of soils varies greatly, ranging in 20 French soils from 4.3 to 69 mg. per kilo dry soil (19), traces also being found by spectrographic analysis in Atlantic sea water.

Response to molybdenum, when it occurs, is shown by increased yield and a deeper green colour of the crop. This is frequently seen in lettuce grown in water culture (32), where 1 part in 10 million of the element is adequate. The response in this crop is uncertain, even when the successive crops are grown under apparently similar conditions, but the reason for the variability is not yet known. Leguminous plants appear to respond well to molybdenum, and Bertrand (20) gives 0.4 mg./l. (= 1:2,500,000) as the optimum concentration. On shaley clay-loam soils in Australia rye grass and clover species responded well to dressings of molybdenum supplying one part of the element per million parts of the dry plant material. Stephens and Oertel (143) also indicate that the availability of molybdenum is greater with alkaline soils than under acid soil conditions. The poor pastures on the ironstone soils of South Australia have been much improved by application of two pounds of sodium molybdate per acre, the yield of lucerne on the same soil being nearly doubled, while the plants were dark green instead of the yellowish colour of those grown without added molybdenum (5). Fricke (59) reports that in field trials on mixed pasture progressively improved growth was obtained with one, two and four pounds of ammonium molybdate per acre and that successful results were also obtained by use of roasted molybdenite. Subterranean clover is quoted as one of the leguminous plants most benefited by molybdenum.

On podzol soils molybdenum alone may be either useless or actively harmful to the yield of clover (47). Both yield and general development of plants were improved when boron and lime were added to the molybdenum, the optimum of the latter element being from 1 to 5 mg. per kg. soil, larger quantities being harmful.

Lyon, Beeson and Ellis (95), working with trace element deficiencies of tomato, found no evident external vegetative symptoms which could be correlated with a limited supply of molybdenum, though there were indications that Mo-deficient plants produced smaller fruits than the controls.

The poisonous effect of excess molybdenum on some plants is well known, oat seedlings being among the susceptible crops (158). Further evidence has accumulated on the part played by molybdenum in the teart disease of animals, and the benefit derived from the administration of 2 gm. copper sulphate per head per day (56). Wild white and wild red clover are exceptionally high in their molybdenum content on such soils, which may help to explain the good response of clover to molybdenum dressings on soils deficient in the element. Teartness may be decreased by use of acid nitrogenous fertilisers which suppress the clovers and also reduce the molybdenum uptake by grasses (91).

Further work has been carried out on the relation of molybdenum to nitrogen fixation. Horner *et al.* (75) found that with optimum quantities of molybdenum various strains of *Azotobacter* showed a ten-fold to thirty-fold increase in nitrogen fixation. The effective range of molybdenum concentration is extremely small, one part per million being the highest level, ranging down to 0.00001 p.p.m. for appreciable effect with *Azotobacter chroococcum*. The range varies with species and with age of culture, being appreciably narrower with young cultures. At the time this work was published it had not been established whether molybdenum is essential for nitrogen fixation by all strains of *Azotobacter*. Jensen and Betty (77), working with lucerne and white clover, showed the need for traces of molybdenum for nitrogen fixation in nodules. In agar culture the ratio between the available molybdenum and the amount of nitrogen fixed was 1:37,000. In sand cultures the growth of lucerne was improved when the ratio of available molybdenum and fixed nitrogen was only 1:80,000, but no further benefit was derived by increasing the molybdenum above a very low level. More

molybdenum was absorbed by lucerne that was dependent on fixing free nitrogen than when combined nitrogen was available for its nutrition. When the molybdenum supply is very low it tends to be concentrated in the root nodules of leguminous plants, comparatively little being passed into the roots and still less into the tops.

An interesting side line on the commercial use of trace elements is the possibility of using them for preservation of cut flowers for market (106). The salts of certain heavy metals, including molybdenum, gave promising results in maintaining petal colour and turgidity when added to water containing sucrose in which the stems of roses were placed during storage.

SELENIUM

Much recent work has been related to the distribution of selenium in soils and its toxic action on various forms of animal life. Special selenium surveys carried out in the United States show the wide distribution of the element, which is probably present in all soils. It has been suggested that the term "seleniferous soils" shall be applied only to areas containing sufficient selenium to produce vegetation which is toxic to animals feeding on it (89).

A wide range occurs in the quantity of selenium present in plants grown on ordinary selenium-containing soils (35). When 5 p.p.m. sodium selenate was added to a normal soil the uptake was very variable, the selenium content ranging from 275 p.p.m. in young maize to 1,240 p.p.m. in mustard, broccoli, spinach, wheat and barley giving intermediate results. Beath (12) reports that species of the same genus vary in their capacity for absorbing selenium. *Astragalus osterhoutii* is confined to seleniferous soils and absorbs selenium in quantities toxic to animals, so much so that the plant is regarded as a hazard to livestock and as contaminating the soil, whereas *A. lonchocarpus* is more generally distributed, contains little selenium and is regarded as a safe forage.

Selenium absorption by plants is markedly increased by the presence of organic matter (151), a fact which may be of importance in the field. Olsen *et al.* (110) found no relation between the selenium content of *Agropyron smithii* and the amount of available selenium in the surface foot of soil, analyses showing that selenium is leached from the surface soil, and obtained chiefly by plants from the second or third foot depth. Trelease (150) found that species

of *Astragalus* indicative of high proportions of selenium in the soil could be determined by germination tests, as such species tolerate or are even stimulated by a high concentration of sodium selenite, whereas non-indicator species are severely poisoned by low concentrations. More evidence is becoming available as to the varying toxicity of identical doses of sodium selenite for different plants (26). Mustard is more adversely affected than millet, and also mustard accumulates a greater amount of selenium from an initial equal supply.

Gill and Lakin (63), working on the effect of soil colloids on the toxicity of sodium selenite to millet, found great variation in effect, and suggest that the total iron content of soil colloids is to some extent correlated with their effect on selenite toxicity. Moist artificial iron gel has a high capacity for reducing the toxicity of selenite. Sodium selenite tends to be more concentrated in plant roots, in contrast to sodium selenate, which accumulates more in the tops. Within the plant tissues the distribution of selenium varies considerably (103). Wheat grown on seleniferous soil had the highest selenium content in the bran, the proportion decreasing with successive reductions of the flour. Selenium is closely associated with the protein of wheat, and as the bran is the fraction richest in nitrogen the high selenium content therein is explicable.

ZINC

The toxic action of excess quantities of zinc in the soil still attracts considerable attention on account of its economic aspect. Industrial wastes from factories distributed over agricultural land may so poison the soil as to devastate the future crops. Absorption of zinc by the plants is heavy—poisoned spring wheat contained 2,500 p.p.m. against 300 parts in normal plants, but the seed from the bad wheat contained only 25 p.p.m., as plants tend to pass on only to the seed the usual constituents (85). It has been suggested that the toxic effect of zinc sulphate on mine dumps may be mitigated by liberal phosphate dressings which would precipitate much zinc and also encourage plant growth (42).

The necessity of traces of zinc for adequate growth, at least in certain cases, is gradually becoming clearer, but the amount required is exceptionally small, and it is difficult to ensure its absence in experimental work. Without any traces of zinc peas and beans

will grow to a certain stage, but will not develop seed (117). Zinc requirements and tolerance vary not only between species but between varieties of the same species. Flax requires more zinc than wheat (102), while Hudson Manchu soy bean will tolerate at least eight times the zinc concentration as the Peking variety (53). Tomatoes (14) and *Pinus radiata* (133) are among the crops in which deficiency has recently been controlled by application of zinc salts. The action of zinc in the soil may be affected by the water content. With red clover an amount of zinc that had a favourable effect when little water was supplied became detrimental as the quantity of water increased (127).

Comparatively little has yet been done on the cytological effect of minor element deficiencies in plants, but by using modern developments in technique Reed and Dufrenoy have demonstrated the nature of certain cellular inclusions which are characteristic of many diseased cells. Spherical inclusions of catechol occur in the cell vacuoles of zinc-deficient specimens of apricot and walnut trees, other pathological conditions in the latter being accumulations of necrotic material and formation of gum in intercellular spaces near severely affected cells (118).

OTHER ELEMENTS

Cobalt. Attention is still focussed on the remedial value of cobalt in cases of pining diseases of sheep and cattle. On grassland in Scotland where pining of sheep occurs, the low cobalt content of the pasture was increased by treatment with cobalt-rich fertilisers, and the disease was as efficiently controlled by feeding on the treated grass as by drenching the animals regularly with cobalt chloride in very small amounts (146, 147). Analyses showed that two pounds of cobalt chloride per acre brought about a marked and speedy increase in the cobalt content of the herbage, which was accentuated by heavier dressings. Similar results were obtained in New Zealand, various cobalt compounds proving equally effective (6, 9, 57).

Fluorine. No evidence has yet been found that fluorine is essential to plant growth. On pastures contaminated by smoke from factory chimneys, the hay had a high fluorine content, and animals fed thereon suffered from chronic fluorosis (100). On the other hand, no injury to germination, plant growth or livestock feeding

on the crops occurred when fluorine was present in the fertilisers used (98).

Iodine. Most recent work on iodine has dealt with analyses of its presence in plants and its effect on animal nutrition. The toxic action of potassium iodide on tomatoes in concentrations above 4 p.p.m. was clearly shown in sand cultures, the ascorbic acid content of the plants also being reduced by all strengths of the iodide (69). In maize the toxic action of iodides is partly mitigated by the presence of chlorides (92).

Lithium. The toxic, rather than the beneficial, properties of lithium are more in evidence in recent work. Meristem cells in the tips of roots of *Zea mais* are easily poisoned, the cytoplasm nuclear material and cell division all being affected (54). Kent, working under the auspices of the Hills bequest, found that diseases of wheat and tomato plants were reduced by application of lithium salts to the soil, a significant inverse correlation existing between the amount of disease present and the quantity of lithium in the soil or in the plant. Higher concentrations which were toxic to the plants reduced the disease still further (82). In wheat plants lithium first accumulates in the roots and then in the oldest leaves. The latter seems to be immobilised and cannot be translocated, though lithium can be lost from the roots to the soil if the lithium gradient favours movement in that direction (83).

Nickel. Complete failure of crops in certain fields in Aberdeenshire (Scotland) was traced to the presence of abnormal quantities of soluble nickel and copper compounds in the soil, approximately 100 times the amounts usually found. Lime and phosphate were used in an attempt to overcome the infertility, and the application of lime proved to have a beneficial effect (96).

Rubidium. This element when absorbed by the roots of barley from rubidium bromide solutions is present in greater concentration at the root apex, more rubidium being accumulated than bromine (145). The former is transferred to the stem more slowly than bromine, and the concentration in the roots continues to increase for a long period. If, however, the source of supply is removed the roots become gradually depleted of rubidium.

Chlorella, grown under conditions of potash deficiency, shows marked decrease in photosynthesis (109). Carbon dioxide assimilation is immediately increased if potash is supplied, or the potash can be replaced by rubidium and to a less extent by caesium.

Thorium. This element may exert a stimulating action on the growth of *Avena sativa* and *Poa pratensis*, which is more marked on loam soils than on sandy soils. Seed development is less benefited than the growth of the stalks (64).

Vanadium. Vanadium has been detected by colorimetric measurements in many plants, very little being present in leguminous seeds (21). Small traces of the element stimulate the growth of *Aspergillus niger* (22) and also increase nitrogen fixation in the roots of *Trifolium pratense* (62).

SUMMARY

In spite of war conditions, work on the relations between minor elements and plants has made considerable progress, and the literature on the subject has greatly increased, especially in regard to boron. Relatively little advance has been made in discovering fresh elements that can be considered as essential, using the word in its strict sense. Attention has rather been focussed on the practical aspects of the problem, such as the improvement in crops brought about by the use of certain elements as subsidiary manures, or the damage done by others in similar conditions. The effect of minor elements, absorbed by the roots, on the nutritive or toxic properties of crops and herbage in relation to animal nutrition has received more consideration, as also has the value of spraying for remedying minor element deficiencies, and for controlling various forms of plant disease. Apart from boron most of the work has related to arsenic, copper, manganese, mercury, molybdenum, selenium and zinc, all of which were recognised before the war as having important relations with plant growth in one way or another. Although so much work has been done, the fundamental problem of the function of minor elements in plant metabolism still remains unsolved, and a vast field remains open for investigation.

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THE ABSORPTION OF ELECTROLYTES IN LARGE PLANT CELLS. II¹

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NATURE OF THE PROTOPLASMIC SURFACE²

Non-aqueous Character of the Surface. It is stated in the previous article (62) that in the plant cells here considered the protoplasm forms a layer not over 10 microns thick. It has an outer non-aqueous surface layer, *X*, and a corresponding inner non-aqueous surface layer, *Y*; between these lies the aqueous layer, *W*.

It is a striking fact that the outer surface acts somewhat like a potassium electrode. For example, when *Nitella* is in contact with 0.01 M KCl, replacement of this solution by 0.01 M NaCl may cause a change of potential in a positive direction of 80 mv. or more (59)³. This has been called for convenience the "potassium effect".

Moreover, when 0.01 M KCl is replaced by 0.001 M KCl there is a change of potential in a positive direction which may amount to 50 mv. or more (59); this is known as the "concentration effect".

Similar results are obtained with *Valonia* (3, 23, 66) and with *Halicystis* (8, 69).

Since it seems improbable that a layer of substance composed largely of water, such as protein in contact with water, can give such values or have the extremely high electrical resistance found in *Nitella* (2, 16, 22), it seems reasonable to conclude that the outer surface layer of the protoplasm belongs to the group of substances immiscible with water which have these electrical properties (1). A variety of evidence indicates that the inner surface also belongs in this category (8, 82, 85).

The protoplasmic surfaces evidently have the physical properties of such substances. When a *Nitella* cell is punctured with a sharp needle and protoplasm comes out, it rounds up in contact with water like an oily liquid. We see the same appearance when the protoplasm is withdrawn from the cellulose wall by plasmolysis.

¹ Supplement to article in *The Botanical Review* 2: 283-315. 1936.

² In the present article it will be assumed for convenience that concentrations are equal to activities in all cases.

³ This change is very rapid and hence must occur at *X*, since apparently there is not time enough for the NaCl to penetrate to *Y*.

The inner protoplasmic surface, Y , also acts like an oily liquid, as is evident in *Nitella* when this surface is rapidly deformed by protoplasmic motion (81).

If the non-aqueous surface layer is not actually liquid but consists of a solid film, it must be so thin as to conform completely to the shape of the liquid which it encloses. It is too thin to be visible under the microscope and hence must be less than half a wavelength of visible light in thickness.

The idea that the protoplasmic surfaces behave like substances immiscible with water is supported by the study of a model (74) in which such a substance replaces the protoplasm. Guaiacol has been employed for this purpose. The model has an artificial sap in which CO_2 is supplied to imitate its production by the living cell.

The model is designed to imitate to a certain extent some characteristic features of *Valonia*, not only in respect to permeability but also in respect to electrical behavior, increase of volume of the sap and accumulation of electrolytes.

Some of the more striking points of resemblance are as follows:

1. The guaiacol resembles the protoplasmic surface in being permeable to water and to strong electrolytes and likewise in being much more permeable to weak electrolytes and to non-electrolytes.

Since it is not necessary to assume a mosaic structure here it does not seem necessary in the case of protoplasm.

It may be noted that Wartiovaara (106) states that water penetrates at about the same rate as heavy water, although cells of *Tolypellopsis* containing heavy water burst when transferred to ordinary water. Heavy water penetrates faster than methyl alcohol. The penetration constant for water which he gives is more than twice that obtained by Palva (89a) who used a method based on specific gravity.

2. In the model as in the cell, an ion may reach a higher concentration inside than outside.

3. There is considerable evidence for the idea that the protoplasmic surface is acid in nature (65), and recent studies on *Nitella* (84) indicate that X contains an acid, possibly a fatty acid, which is washed out more rapidly by KOH than by NaOH , as happens with the fatty acids in ordinary soaps. After treatment with KOH the behavior changes so that a dilute solution of KOH becomes electrically positive to a more concentrated solution (positive cell) instead of the reverse condition (negative cell) found before treatment.

In the model the layer of guaiacol is acid in character and bases enter by combining with it, as appears to be the case in *Valonia* (30, 61).

4. The order of penetration in the model is potassium > sodium > magnesium, calcium, and also chloride > sulfate, as in *Valonia* (62) and in certain other cells.

5. Water and electrolytes enter until a steady state is reached; the artificial sap then increases in volume without change in composition, as in *Valonia* and in many other cells.

6. A difference of about 2 pH units is maintained between the sap inside and the external solution, as in *Valonia*. This happens because CO₂ is supplied to the artificial sap.

7. A difference in the concentration of KOH inside and out is maintained which is about the same as in *Valonia*.

8. A steady potential is maintained. This is outwardly directed, as in some *Valonia* cells, although in *Valonia* it is more commonly directed inward. In *Nitella* and *Halicystis* the potential is outwardly directed.

9. The electrical behavior of inorganic ions can be changed by organic substances, e.g., by nitrobenzene (79). This also occurs in the protoplasmic surface of the living cell. For example, such changes are produced by guaiacol in *Nitella* (68, 70, 72), *Valonia* (63, 64) and *Halicystis* (67); by aniline (76), benzene (75) and nitrobenzene in *Valonia* (75); and by hexylresorcinol in *Nitella* (71, 72).

10. The model shows a potassium effect of 15 mv. which equals what is found in some *Valonia* cells, although the average for *Valonia* is about 50 mv. (23). But the value in the model can be raised by adding nitrobenzene to the guaiacol, and in a model with nitrobenzene in place of guaiacol (79) the potassium effect is 67 mv., which compares favorably with that found in *Nitella* (this ranges from 30 to 95 mv.). In *Halicystis Osterhoutii* (66) it is 68 mv. and in *H. ovalis* (3) it is 45 mv⁴.

11. The potentials in the model (78) appear to be chiefly diffusion potentials, and this seems to be true of the living cell. The observed potentials in the model agree closely with the calculated values.

⁴ In *H. ovalis* the value is sometimes greater than 45 mv. (L. R. Blinks, personal communication).

In *Valonia* and *Halicystis* the values are obtained by substituting for sea water an artificial sea water in which Na is replaced by K.

If we treat the model as we treat the living cell, by calculating relative mobilities from the observed potentials, we get approximately correct values. This indicates that the values obtained for the living cell have real significance.

Such models have a surprising degree of flexibility, recalling that of the living cell. They not only show the importance of partition coefficients in penetration but indicate how changes in the non-aqueous surfaces of the protoplasm may cause alterations in permeability and produce injury and death.

Since in the living cell the non-aqueous surface layer rests on a substrate which is a sol or a gel composed of protein and other substances, we may suppose that anything which alters this sufficiently may bring about irreversible changes in the non-aqueous layer which may result in death.

It has been pointed out (103) that the presence of an unstirred layer in the liquid adjoining the protoplasmic surface will serve to delay the passage of solutes (see (25, page 222)).

Differences between Internal and External Protoplasmic Surfaces. It has been shown by electrical measurements that X and Y behave differently. Further evidence of this divergence is furnished by the differential action of certain reagents⁵. Formaldehyde (82) and mercuric chloride (83) affect Y sooner than X if X is in contact with 0.001 M NaCl, but if X is in contact with 0.01 M KCl it is affected before Y . Furthermore salts like NaCl, when applied in high concentrations, may penetrate X more rapidly than Y . Hence they raise the osmotic pressure in W which in consequence expands by taking water from the vacuole (80). If this took place by ionic exchange it would be necessary for the protoplasm to produce in a few minutes enough new ions to carry on the exchange, but in that case the shrinkage of the vacuole could be produced by these new ions without any penetration of NaCl. If we assume that NaCl penetrates without ionic exchange (presumably by forming molecules at the outer surface of the protoplasm) the difficulty disappears.

Effects of Distilled Water on the Protoplasmic Surface. Previous papers stated that distilled water may remove from the protoplasmic surface of *Nitella* a substance which is responsible for the potassium effect and for the transmission of stimuli. Recent results show that

⁵ Earlier experiments showed that $MnCl_2$ affects Y more than X in *Valonia* (37). In *Valonia*, sap is toxic when applied externally (57).

the normal condition of the surface can be promptly restored by application of guanidine (73, 77)⁶ and more slowly by certain inorganic salts (86).

This indicates that the surface layer of the protoplasm contains more than one substance, since a substance or group of substances can be removed from it without killing the cell.

The Surface Is Not a System of Electrically Charged Pores. Electrical evidence shows that *X* is not a pore system, for, in some cases at least (64, 67), the order of ionic mobilities in *X* is $K^+ > Cl^- > Na^+$. This shows that we do not have to do with a system of electrically charged pores, since in that case all cations would be faster than anions or *vice versa*. (See also 84.)

This does not conflict with the idea (18) that the molecular size of non-electrolytes may influence their penetration, since the smaller molecules may diffuse faster in the non-aqueous layer and elsewhere. The fact that substances of high molecular weight, such as neutral red (20a), may penetrate rapidly is doubtless due to high partition coefficients. The lack of penetration of acid fuchsin, orange G and cyanol (20b) may be due to low partition coefficients.

The order of entrance of electrolytes varies considerably. In *Valonia* and in *Halicystis ovalis* the order is potassium > sodium > calcium, magnesium and chloride > sulfate (62). This is also the order in several species of Characeae (17). See also (19a). Studies (19) on one of the Characeae (*Tolypellopsis*) give the order as potassium > rubidium > calcium > sodium, > lithium. Caesium occupies a low position in the series, as seems to be quite generally the case (21, 29). In *Halicystis Osterhoutii* sodium and potassium enter at about the same rate (62).

Study of the protoplasmic surface is greatly hampered by our ignorance of surface phenomena in general. It is most desirable to know more of the behavior of very thin non-aqueous films formed between two aqueous solutions. A beginning has been made in this field (*cf.* 24-26, 74, 101, 108).

It may be added that the permeability of the protoplasmic surfaces may afford the best test of death that we possess. As long as the non-aqueous surface is intact, such a dye as acid fuchsin does not enter the cell to any noticeable extent, but when it becomes sufficiently permeable to admit acid fuchsin freely the alteration appears

⁶ Guanidine increases irritability (77).

to be irreversible and the cell never recovers its normal impermeability; it soon disintegrates. In a *Spirogyra* cell the color of the cell sap after entrance of the acid fuchsin does not differ much from that of the colored solution outside. On the other hand, in the unfertilized egg of the marine worm, *Nereis limbata*, the color after death of the egg becomes much deeper than that of the external solutions of acid fuchsin because the dye combines chemically with the protoplasm of the egg.

KINETICS OF PENETRATION

If protoplasmic surfaces consist of substances immiscible with water they presumably have low dielectric constants and hence permit relatively little dissociation so that electrolytes pass through them chiefly in molecular form.

A strong electrolyte may form molecules at the outside of the surface layer of the protoplasm⁷ and then pass through in molecular form and dissociate on reaching the aqueous solution on the other side. But we can not predict precisely in what form electrolytes will enter. If we place NaCl outside, the entering molecules may be NaCl, NaOH or HCl.

Molecular transport through the surface must depend chiefly on the mobility of the molecule in the non-aqueous surface layer and on the number of molecules entering this layer, and this, in turn, in the case of strong electrolytes, will depend on the rate at which molecules are formed from ions at the outer surface of the non-aqueous layer. We can not assume that all strong electrolytes form molecules to the same extent at the surface of the non-aqueous layer. For example, potassium and sodium guaiacolate are strong electrolytes in water but on passing into guaiacol become weak electrolytes, and the former produces relatively more molecules than the latter⁸.

Since in strong electrolytes the rate of entrance of molecules depends on the number of collisions by which the molecules are formed. In the case of a strong acid, HA , we might suppose that if there is no dissociation in the non-aqueous surface layer and the number of molecules formed is proportional to the product $(H)(A)$,

⁷ This happens, for example, when potassium guaiacolate enters guaiacol (90). An analogy is seen when HCl passes from water to air.

⁸ The dissociation constant in guaiacol of Na-guaiacolate is 2.8×10^{-5} and that of K-guaiacolate 3.4×10^{-5} (90).

we should find that doubling the concentration would increase the rate of entrance four times. But this can not be expected, since there are other variables to be considered. Among these are competition for available space at which to enter and competition for carriers in the surface layers⁹. At each of these layers (inner and outer) there are two partition coefficients to consider (14a, 29b, 29c) which may differ if the adjacent aqueous solutions differ.

In ionic exchange there are several factors to be considered. The rate of exchange depends on the mobility of the slower ion (60) and on the number of ions which in turn depends on the dissociation constant and the number of molecules. Electrical effects also play a part (109) (see also 25, 48, 49, 50, 51, 100).

It is evident that we can not define the permeation constant of a single ion without specifying just which ions enter into the exchange.

By electrical measurements we can determine relative ionic mobilities in the outer protoplasmic surface and in some cases (where perfusion of the sap is possible) at the inner surface (8). They also give us information regarding partition coefficients. For example, they show considerable variation in partition coefficients in *Nitella* (27). This implies a corresponding variation in the protoplasmic surface.

An interesting effect which may be due to partition coefficients is seen in the behavior of potassium isotopes. Since Brewer (9, 10, 11, 47) had reported that in certain cases the ratio $K^{39} \div K^{41}$ in the organism is not the same as in the environment, Jacques (42) sent him samples of the sap of *Valonia* and of *Nitella*. In general, the value found in nature of $K^{39} \div K^{41}$ is 14.20, and this is true of Bermuda sea water. In *Valonia* sap it is 13.85 and in *Nitella* sap it was 13.85 in one sample and 14.00 in another. These figures are regarded as significant by Brewer. This result can hardly be due to differential diffusion, since the diffusion path is very short, but it might possibly be due to a difference in partition coefficients. It has been suggested that more KCl than NaCl is taken up by *Valonia* because the partition coefficient of KCl is higher, since it is higher in the non-aqueous surface layer in guaiacol models (62). This is in accordance with the rule (91, 92) which states that the partition coefficient increases with the ionic radius. On this basis

⁹ In bases there is also competition for the carrier in the protoplasmic surface (30).

we might expect compounds of K^{41} to have a higher partition coefficient than those of K^{39} . Determinations indicate that this may be true of guaiacol (42).

Penetration of strong electrolytes into sap is relatively slow. Thus the penetration of NaI into the sap of *Valonia* is one millionth as rapid as through a layer of water (or of gelatin imbibed with water) of the same thickness as the protoplasm (33). Similar values have been obtained for the exit of sodium from members of the Characeae (19).

Brooks has observed very interesting results (13-15, 52, 53) by placing cells of *Nitella* in solutions containing radioactive substances. For example, radioactive K^+ and Rb^+ enter the protoplasm rapidly, and the number of ions in unit volume may quickly become much greater in the protoplasm than in the external solution. This is regarded as the result of an exchange of the ions (largely combined) in the protoplasm with those in the external solution.

After an initial period of intake the protoplasm loses ions for a time. This is followed by a period of slow steady intake. In addition to these changes smaller fluctuations may occur.

The exit of radioactive ions into distilled water is relatively slow. It is somewhat more rapid when the external solution contains alkali chlorides. When radioactive K or Rb is coming out, the rate decreases as the atomic weight of the alkali cation outside decreases, so that the series is $Cs > K > Na > Li$, but when radioactive Na is coming out, the order is reversed, so that we have $Li > Na > K > Rb > Cs$ (54, 55).

Mullins (53) finds that radioactive K is preferentially taken up by certain protoplasmic granules. Wernstedt (107) obtained similar results with radioactive lead.

In striking contrast to the rapid penetration of radioactive ions into the protoplasm is the considerable delay in their appearance in the sap. Hence Brooks considers that the chief resistance to the penetration of electrolytes is located in the inner protoplasmic membrane surrounding the sap. Later experiments on the penetration of radioactive K and Na into *Nitella* and *Tolypellopsis* by others (29a) led them to locate the chief resistance in the outer protoplasmic surface.

Brooks has developed a detailed theory to explain the entrance of electrolytes, but as this can not be adequately summarized here the reader is referred to his exposition (12a, 15).

It is evident that permeability is dependent on a variety of factors. Some further effects may now be considered.

Effect of Light. Light may effect permeability and supply energy: photosynthesis supplies material for the growth of the cellulose wall, raises the pH (8) and supplies oxygen (8). Light may decrease electrical resistance in *Halicystis* (6) after resistance has been increased by lack of oxygen. Collander (19) states that light increases the penetration of electrolytes in *Chara* and in *Tolypellopsis*. It is claimed (46) that light (especially of shorter wavelength) increases the rate of intake of certain non-electrolytes in *Chara*. This effect can not be due to supply of energy, since there is no accumulation and it does not seem probable that pH is concerned. But there may be a direct effect on permeability or on the cellulose wall.

The equilibrium concentration of NH_3 in *Valonia* is about twice as great in light as in darkness (37), and it is found that the rate of penetration of NH_3 in light is greater than in the dark. Jacques states that light promotes both the entrance (37, 38, 41)¹⁰ and exit of NH_3 and in impaled *Halicystis* accelerates the entrance of water and of electrolytes. The action of light on the pH inside and outside can apparently account for the following facts. In dim light potassium comes out as NH_3 goes into *Valonia*, and if NH_3 has previously been stored in the sap we find that when it comes out sodium enters but not potassium. But in stronger light potassium enters, apparently because the pH outside is increased by photosynthesis more than that inside (34).

Blinks (7) found that the effect of light on potentials in *Valonia ventricosa* increased as the potassium content of the sea water increased.

Effect of Temperature. An exposure of several days to a temperature of 5° C. increases the resistance and capacity of *Halicystis* (8). The effect is reversible. It may perhaps be due to the production of weak acids inside the cell.

Wartiovaara (105) finds in *Tolypellopsis* very high temperature coefficients (up to 8.9 for Q_{10}) for the penetration of substances of high molecular weight to which the cells is highly permeable.

Effect of Oxygen. Blinks finds that in an atmosphere of nitrogen (containing 0.2% oxygen) the electrical resistance of *Halicystis* is

¹⁰ In the exit of NH_3 there may be an induction period of a day or more; this is shorter in light.

greatly increased (8). The effect promptly disappears when oxygen is restored or when the cells are given sufficient light to permit photosynthesis to produce oxygen.

Collander (19) finds that lack of oxygen may somewhat inhibit absorption of lithium in certain Characeae. According to Blinks (8), *Nitella* is not sensitive to lack of oxygen, since after some hours in nitrogen or hydrogen it shows no change in respect to stimulation and propagation of stimuli.

Effect of Weak Acids. The effect of weak acids is very much like that of lack of oxygen (8) in causing a great rise in resistance.

Blinks suggests that the rise of resistance which occurs during recovery after an action current in *Nitella* may be due, in part at least, to the production of CO_2 which occurs at that time (8).

A model of the effect of acids on resistance has been devised which gives increases comparable to those found in the living cell (74).

Effects of Electrical Current. Experiments have been made (98) in which cells of *Chara* were placed in 0.5% cyanol or orange G (which do not enter the living cell) and subjected to an electric current, but this did not cause the dyes to enter. These results were opposite in character to those obtained by others with animal cells.

Effects of Concentration of Electrolytes. Experiments with impaled *Halicystis* in dilute sea water give interesting results (39). As dilution progresses, the permeability constant for the entrance of water falls off gradually until the concentration of the sea water is reduced by one half. Further dilution results in a rapid falling off in the permeability constant. In most cells dilution of sea water to 50% produces injury, and hence we might expect an increase of permeability.

Effect of the Cellulose Wall. In dealing with penetration into plant cells it is customary to consider only the permeability of the protoplasm. Although the mechanical restriction of the cell wall may be a most important factor, its effect is usually neglected.

When the restrictive action of the cellulose wall is removed by piercing it with a capillary into which the sap can move (36, 40), the rate of entrance of electrolyte and of water is increased 10 times in *Halicystis* and 15 times in *Valonia*. It is, therefore, evident that any factor which changes the rate of growth of the cellulose wall may affect the rate of entrance.

Under normal conditions water can not enter faster than the cell

wall expands. When the restriction of the cell wall is removed, water enters more rapidly and causes a corresponding dilution of the cell sap.

As a result of this electrolytes enter more rapidly. It is not necessary to assume any increase in permeability to electrolytes, for the rate of increase in entrance of electrolytes corresponds exactly to that of water, and it is not necessary to assume any increase in permeability to water.

With the restriction of the cellulose wall removed the situation may be compared with that in a guaiacol model (89).

We may now consider some additional statements which bear upon permeability.

The entrance of H_2S into *Valonia* appears to be practically all in molecular form. This is shown (31) by measuring rates of penetration. It is found that the rate of penetration is directly proportional to the external concentration of undissociated H_2S regardless of the external concentration of dissociated H_2S (HS^- and $S^{=}$).

The curve of penetration may be regarded as of the first order with a constant decreasing somewhat with time.

The fact that H_2S enters chiefly in molecular form is also indicated (58) by conditions at "equilibrium" (which is really a steady state, since the pH is not the same inside and outside). If H_2S enters in molecular form and dissociates inside, the pH of the sap will be lowered and this effect will be at a maximum at "equilibrium", as is actually the case. If sulfide ions entered to any great extent in exchange for Cl^- coming out, they would combine with H^+ inside and raise the pH; this does not happen.

Lithium is taken up relatively faster by *Chara* from dilute solutions. The rate of absorption increases from two to four times when the concentration in the external solution increases ten times (19).

There is some evidence that potassium enters *Valonia* chiefly (43) as KOH , but this is not supported by experiments on *Nitella* (32), impaled *Halicystis* (41), *Chara* or *Tolypellopsis* (19), since in all these the rate of entrance of potassium appears not to be influenced by the pH of the external solution.

Interesting results have been obtained in experiments with nitrate (35). In sea water the ratio of Cl to NO_3 is 80,000 to 1, and in the sap of *Valonia macrophysa* the average value is 18 to 1. When Cl in the sea water was replaced by NO_3 so far that the ratio Cl to NO_3

became 1.75 to 1, the rate of entrance of Cl became 1.68 times that of NO_3^- . No Cl came out in exchange for NO_3^- . It may be noted that the mobility of NO_3^- in the outer protoplasmic surface of *Halicystis* is less than that of Cl^- (8), but this does not mean that KNO_3 would necessarily enter more slowly if the flux were chiefly in molecular form.

We can not make any precise prediction regarding entrance of ions into the sap by knowing ionic mobilities in the outer non-aqueous layer. For example, in *Halicystis Osterhoutii* the mobility of K^+ in the outer protoplasmic surface is about 80 times that of Na^+ , but the relative rate of penetration into the vacuole is about the same for potassium and for sodium (74).

In *Halicystis ovalis* the relative mobility of K^+ as compared to Na^+ in the outer protoplasmic surface is less than in *H. Osterhoutii*, but in the former the rate of entrance of potassium into the sap is much higher, as judged by the composition of the sap (8).

It is evident that permeability is affected by many variables, and it is not safe to generalize from one organism. For example, *Valonia* (62) takes up potassium in preference to sodium, but *Halicystis Osterhoutii* (62) does not, although *Halicystis ovalis* does (12).

It may be added that great temporary increases of permeability are possible, as in the stimulation of *Nitella* (5, 16) and in the delayed polarization state of *Valonia* (4). When the cell becomes completely and irreversibly permeable we may regard it as dead, and this appears to be the best test of death which we possess (56).

ACCUMULATION¹¹

Certain electrolytes entering the cell attain a higher activity inside than outside. This involves an expenditure of energy on the part of the cell and a storage of potential energy in the cell. Apparently energy in electrical form is involved, since this process appears to occur only with electrolytes (18, 20, 46). This may be called "true accumulation".

¹¹ According to some workers (93, 94, 97), there is one type of absorption of ions found in all tissues, which Steward calls "induced" accumulation; here ions previously acquired are given up in exchange for available external ions. This "induced" accumulation does not increase the salt content of the tissue.

Steward designates as "primary" accumulation that which occurs only in tissues capable of growth and is connected with absorption of oxygen and metabolism.

We may, for convenience, designate as "apparent accumulation" the process by which an ion reaches a higher activity inside than outside without any expenditure of energy¹². An example is seen in the Donnan equilibrium which involves a run-down of energy to reach the equilibrium state. If, for example, all the indiffusible ions inside have a positive sign, there will be at equilibrium more negative diffusible ions of any species inside than outside. There will be no excess activity inside of any diffusible compound.

The laws governing the Donnan equilibrium are sometimes applied to biology without considering that they are based on the assumption that water is not allowed to move. If water moves it will enter the cell and upset the equilibrium.

This is evident from a simple calculation. Let us assume that the cell contains an indiffusible ion A^- and the diffusible ions Na^+ and Cl^- , the concentration of NaA being 1.8 M and that of $NaCl$ 0.6 M; also that we have outside a very large volume of $NaCl$ 1.2 M. We assume that water does not move.

To fulfill the conditions of the Donnan equilibrium we must have $(Na)_i(Cl)_i = (Na)_o(Cl)_o$ where the subscripts i and o denote inside and outside. In this case we have $(Na)_i(Cl)_i = 2.4(0.6) = 1.44$ and $(Na)_o(Cl)_o = (1.2)(1.2) = 1.44$.

The total molar concentration inside is $NaA = 1.8$ and $NaCl = 0.6$, *i.e.*, total 2.4. Outside the total molar concentration is $NaCl = 1.2$. Hence the osmotic pressure inside is higher. Let us suppose that water (but not salt) now moves and that water enters until the osmotic pressures inside and outside become equal. We then have inside NaA 0.9 M and $NaCl$ 0.3 M or a total molar concentration inside of 1.2 M. But now $(Na)_i(Cl)_i = 1.2(0.3) = 0.36$ which is less than the external product.

Such calculations indicate that if water can move without restriction both water and salt will continue to enter until no external solution remains.

The entrance of water into plant cells is considerably restricted by the cellulose wall. It may be added that no plant cells hitherto examined yield evidence of Donnan equilibrium (62).

The Donnan effect may be partially achieved if in place of indiffusible ions we have slowly diffusing ions whose activity is maintained by metabolism (102).

¹² This happens in certain models (87, 88, 89).

Apparent accumulation is seen when a weak base MOH enters a plant cell where metabolism keeps the internal pH lower than the external. Thus if the activity of OH is 10^{-8} inside and 10^{-6} outside, MOH may enter until the ionic activity product (M)(OH) is the same inside and outside, that is, until the activity of M inside is 100 times as great as outside, but the chemical potential and the activity of MOH will not become greater inside; see, for example, the experiments on dyes (62).

In certain models where the artificial sap is supplied with CO_2 to imitate its production in the living cell, the activity of K^+ reaches a much higher value inside than outside, and under certain conditions the value of the ionic activity product (K)(Cl) may become greater inside than outside (62, 87, 88, 89).

In this connection mention may be made of a model set up by Teorell (99) while investigating the cause of the acidity of gastric juice. A buffered solution of sodium benzoate (inside) of pH about 7 is separated from a solution of $NaCl$ (outside) by a layer of CCl_4 which is not permeable to Na^+ . Benzoic acid passes out and lowers the pH of the solution of $NaCl$ outside to about pH 4 in two hours while the pH inside shows little change.

True accumulation produces an excess of electrolyte and hence of osmotic and of hydrostatic pressure in the sap which gives the cell turgidity.

It would seem that an automatic mechanism keeps the excess of electrolyte in the sap within certain limits. The excess of halide is about 0.05 M with *Valonia macrophysa* in sea water and in certain hypertonic solutions (35); in *V. ventricosa* it is about the same (95, 96); and in *V. ocellata* it is somewhat higher (95). In *Halicystis Osterhoutii* it is about 0.023 (40). In *Hydrodictyon* (8a) it is 0.054. The excess of internal osmotic pressure in some cases in *V. macrophysa* is about 1.71 atmospheres and in *Halicystis Osterhoutii* about 0.38 atmosphere (40)¹³. In *Nitella flexilis* it is about 6 atmospheres at 25° C.

The osmotic pressure in these cases is practically all due to electrolytes and hence may be regarded as due to true accumulation in

¹³ In *Valonia macrophysa* the difference in freezing point depression between sea water and sap is 0.129° C. Using the formula, pressure in atmospheres = 0.04416 T (ΔT) where T is absolute temperature and ΔT is the freezing point depression, we have at 27° C. the value 0.04416 (300) 0.129 = 1.71. For *Halicystis Osterhoutii* we have 0.04416 (300) 0.029 = 0.384 atmosphere.

contrast to certain cases (as in the sugar beet) where the osmotic pressure is raised by organic substances produced in the cell.

In *Nitella clavata* the excess of halide inside is 0.09 M (28) and in *Nitella flexilis* it is about 0.1 M. Here organic substances may contribute.

The ratio of iodide (44) in the sap to that in the sea water is from 1,000 to 1 up to 10,000 to 1 in *Halicystis* and from 40 to 1 up to 250 to 1 in *Valonia*. There is true accumulation of KI, NaI and HI. In *Valonia* the concentration of NO_3 in the sap is at least 2,000 times that in sea water and in *Halicystis* at least 500 times that in sea water. Hence we have true accumulation (45) of KNO_3 , NaNO_3 and HNO_3 . For other ratios see tables in the previous publication (62).

Collander (17) analyzed the sap of several species of Characeae growing in fresh and in brackish water. He found that five species in fresh water showed an excess of Cl^- inside varying from 0.108 to 0.193 M and that three species in brackish water showed an excess of Cl^- inside of about 0.156 M (three other species did not diverge greatly from this value).

Investigating the same species of *Chara* growing in fresh and in brackish water he found in the former an excess of cations ($\text{K}^+ + \text{Na}^+ + \text{Ca}^+$) amounting to $0.174 - 0.004 = 0.170$ and in the latter to $0.228 - 0.073 = 0.155$ equivalent per liter. The corresponding values for Cl^- are $0.176 - 0.00013 = 0.176$ and $0.232 - 0.08 = 0.152$. In the former the ratio Cl^- inside \div Cl^- outside is 1,350 and in the latter 2.9. As the external concentration of Cl^- increases 615 times, the internal concentration increases only 1.3 times.

Evidently the difference between internal and external concentrations tends to remain constant but not the ratio of internal to external concentration.

This might be expected if there is a mechanism which keeps the excess of electrolyte inside within definite limits. It might be supposed that the excess of internal osmotic pressure would have to reach a certain value in order to cause the cellulose wall to expand and allow water to enter, and in growing cells the internal excess would be thus determined. But this does not seem to be the case. When this restriction is removed by puncturing the cellulose wall with a capillary into which the sap can move water and electrolytes enter *Valonia* (36) about 15 times as fast as under normal condi-

tions. In *Halicystis* (40) the rate increases 10-fold and continues undiminished for five days or more; in *Valonia* it continues until the capillary becomes plugged (in some cases for more than two days). But the composition of the sap remains approximately the same as in normal cells and the concentration is greater than that of the external solution. Hence it is evident that the excess of osmotic pressure inside does not depend on the cellulose wall¹⁴. If a cellulose wall is present this excess of osmotic pressure produces turgor.

The facts cited here indicate that the pumping action is able to produce a certain excess of electrolytes inside and that this excess does not differ greatly in fresh water, brackish water and sea water.

TABLE I

RATIO OF CONCENTRATION IN THE SAP DIVIDED BY CONCENTRATION OUTSIDE IN FOUR SPECIES OF CHARACEAE GROWING IN FRESH WATER (FINLAND)

	<i>Chara ceratophylla</i>	<i>Tolypellopsis stelligera</i>	<i>Nitella flexilis</i>	<i>Nitella gracilis</i>
Cl	294 1350	1150 980	1300 3300	1600
Na	122 400	100 90 390 31
K	650 1900	3200 4200	2500 1900	13000
Mg	50 9	42 40
Ca	10 4	15 7	42 77 49

NOTE: Where two values are given they refer to different localities (these are different for different species).

The excess of internal electrolyte may be limited by the amount of energy available to produce it.

Some accumulation ratios (17) are shown in Tables I and II.

Work on *Hydrodictyon* (8a) brings a welcome addition to the list of plants with sap of known composition. (*Hydrodictyon* belongs to the Chlorococcales, *Nitella* and *Chara* to the Charales, *Valonia* to the Siphonocladiales, and *Halicystis* to the Siphonales.)

The results obtained with *Hydrodictyon* are shown in Table III.

¹⁴ In a guaiacol model (89) where there is no restriction corresponding to that of a cellulose wall, the internal excess is automatically regulated in the sense that addition of electrolyte to the artificial sap is followed by an increased intake of water which soon restores the concentration of electrolytes in the sap to the value existing before the addition was made.

TABLE II

RATIO OF CONCENTRATION IN THE SAP DIVIDED BY CONCENTRATION OUTSIDE IN SIX SPECIES OF CHARACEAE GROWING IN BRACKISH WATER (FINLAND)

	<i>Chara ceratophylla</i> *	<i>Chara baltica</i> *	<i>Tolypella nidifica</i> *	<i>Nitella hyalina</i> †	<i>Chara ceratophylla</i> ‡	<i>Tolypellopsis stelligera</i> ‡
Cl	2.90	2.95	2.94	3.68	5.77	5.64
Na	2.18	2.12	0.81	2.26	4.06	1.32
K	49.3	41.4	76.4	67.6	102.0	233.0
Mg	1.86	3.07	1.38
Ca	2.89	7.63	2.89	3.33	5.50	6.0

* Locality A (external concentration of Cl=0.08 M).

† Locality B (external concentration of Cl=0.05 M).

‡ Locality C (external concentration of Cl=0.036 M).

The osmotic pressure of the sap is approximately equivalent to that of 0.08 M NaCl. Since *Hydrodictyon* grows in fresh water, this represents a considerable excess of internal osmotic pressure.

The problem of accumulation is of profound interest. We know that the cell has energy at its disposal, but the manner in which it is applied to produce true accumulation is completely unknown.

TABLE III

AVERAGE COMPOSITION OF SAP OF *Hydrodictyon patenaeforme*, POCCOCK
Moles per liter

	S = sap	P = pond water	S ÷ P
K	0.0763	0.000019	4000
Na	0.004	0.0013	3
Ca	0.0018	0.00108	1.65
Cl	0.0553	0.00108	51
SO ₄	0.0082	0.00077	10.7

pH of sap 5.5 to 6.0.

Little or no organic matter in sap.

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IMPROVEMENTS IN PLANT CYTOLOGICAL TECHNIQUE. II¹

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¹ Supplement to article in *The Botanical Review* 3: 241-258. 1937.

INTRODUCTION

The ten years that have elapsed since the previous review on this subject (39) have seen a remarkable change in technique. In this period, sections have been largely replaced by smears and squashes to give greater efficiency with saving in time and labour. This change in practice can be largely attributed to the acetic-stain and Feulgen reaction, for it is around these two techniques that present-day methods have been developed.

FIXATION

With these changed conditions it is necessary to reconsider some aspects of fixation. Wide application of the Feulgen reaction now makes possible the use of Carnoy type fixatives hitherto unsatisfactory except for use with acetic-stains. These alcoholic fixatives are particularly valuable because of their rapidity of penetration; they can be used, with some reservation, in all smear and squash techniques.

For large chromosomes in root tips prepared by the paraffin method, osmic acid fixatives give, as a rule, the best results. In Feulgen root-squashes for chromosome counts alone, Navashin fluids or acetic alcohol are less expensive and sufficiently good. With acetic alcohol, the acetic acid can be replaced by propionic acid to give equally good and sometimes better results (*cf.* 36). But where morphological details are of major importance, osmic acid fixatives, and sometimes Navashin fluids, are best. In the study of chromosome structure, *i.e.*, cold starved heterochromatin, fixatives low in acetic acid give the most satisfactory results. This is also true of nucleoli where, for successful application of the light-green technique (Schedule 5), Semmens and Bhaduri (58) recommend either Levitsky's modification of Navashin fluid or a similar fluid in which the formalin is replaced by a uranium salt (5).

According to Johansen (36), the writer's fixatives 2BE and 2BD give atrocious results on the Pacific Coast. He claims that the tissues become overtanned and difficult to stain. In England, at least in the hands of capable workers, no such difficulty is experienced. In this laboratory, although the combination of chromic acid and bichromate may appear illogical, the results obtained with these fluids are not infrequently superior to those obtained with any other fixing fluid.

The following recommended fixative formulae, except where indicated, are given in an earlier review (39):

Feulgen Smears and Squashes

(a) Smears of pollen-mother-cells and pollen grains: Medium Flemming, Benda, La Cour 2BD, Navashin, acetic alcohol (1:3) and sometimes Carnoy (6:3:1). For spiral structure: acetic alcohol (1:2). For nucleoli: Semmens fluid (see formulae).

(b) Squashes of pollen-mother-cells and pollen grains: Acetic alcohol (1:3), Carnoy (6:3:1) and sometimes aqueous fixatives.

(c) Somatic squashes. For chromosome counts: acetic alcohol (1:3). For chromosome morphology: Medium Flemming, Benda, La Cour 2BD and 2BE, Navashin. For nucleoli: Navashin (Levitsky's modification, see formulae).

Paraffin Method and Smears for Crystal Violet Staining

Any of the aqueous fixatives given above.

FIXING TECHNIQUES

The Permanent Smear

The Taylor method described in detail by La Cour (39) still remains one of the most satisfactory means of fixing pollen-mother-cells and pollen grains. It is now more valuable than ever for pollen grains, since with acetic alcohol fixation and Feulgen staining the walls remain unstained, and the cells can be flattened to spread the chromosomes. Also, where the walls are thick and persistent, they may rupture, thereby allowing the cytoplasm to escape entire and undamaged.

Schedule 1. *Feulgen Smears*

Tissues: Pollen-mother-cells and pollen grains

1. Smear with flat-honed scalpel in the usual way.
2. Fix in acetic alcohol (1:3) or Carnoy (6:3:1) 1 hour.
3. Harden in 95% alcohol 6-24 hours (maximum time for meiotic prophase).
4. Drain off excess surface alcohol and dry undersurface of the slide.
5. Hydrolyse in N.HCl at 60° C. 6 minutes.
6. Stain in leuco-basic fuchsin 2 hours.

7. Bleach in SO_2 water, 3 changes 10 minutes in each.
8. Rinse in 45% acetic acid 1-2 minutes.
9. Apply albumenized cover slip² and press lightly by blotting, avoiding lateral movement of the slip.
10. Heat very slightly over a spirit flame 2 or 3 times.
11. Separate slide and cover slip by inverting in a ridged dish containing 40% alcohol.
12. Pass slide and cover slip through 80% and absolute alcohol 2 changes, 2-3 minutes in each; recombine by mounting in Euparal or Diaphane.

Notes: (i) Where aqueous fixation is required, fix 2-4 hours; wash in running water 10 minutes (bleach if necessary in 1 part H_2O_2 : 3 parts 80% alcohol, 1 hr.); omit step 3; hydrolyse 10 minutes.

(ii) With alcoholic fixation, except in a specific test for desoxyribose-nucleic acid, omit step 7.

(iii) The stain can be intensified if needed by rinsing in running water, after step 6, 10 minutes.

(iv) Where flattening of the cells is not required, omit steps 8, 9, 10 and 11.

(v) Where pollen grains are prone to collapse, mount from absolute alcohol into cedarwood oil.

Stain-Fixative Smears

This method provides the simplest means of fixing and staining the chromosomes in pollen-mother-cells and pollen grains. Until recent years Belling's iron aceto-carmine (39) was the only combined stain-fixative in common use. The writer has introduced two new chromosome stains for use in 45% acetic acid *viz.*, orcein (40) and lacmoid indicator (resorcin blue, 20). Like carmine, they stain the chromosomes red and appear to be as permanent. With lacmoid, however, mounting in neutral balsam must be avoided; otherwise the colour changes to an unsatisfactory blue. It remains an intense red in thick Cedarwood oil or Euparal that has become acid with keeping.

In assessing the value of these stains, it is not suggested that they can replace carmine; they will, however, give with many plants intense and more selective staining. Workable stains can also be made from all combinations of all three.

² Dried by passing over a spirit flame.

With all acetic-stains the staining of extremely small chromosomes sometimes proves troublesome. Where this difficulty is experienced, it may be overcome by one of the following:

(a) Thomas (69) with carmine finds pre-mordanting satisfactory. The iron in acetate form is introduced into the acetic alcohol. He finds a weak stain more satisfactory, about 1/3 strength aceto-carmine diluted with 45% acetic acid. If needed, additional iron can be introduced at the time of teasing by using steel needles.

(b) Nebel (48) recommends addition of chloralzol black E as a combined stain with carmine.

Other developments in the acetic-stain technique are:

(i) With large chromosomes that are not sufficiently spread, interpretation of chiasmata may prove difficult. This can be overcome by enzyme treatment to soften the cytoplasm (26). It consists in treating a piece of anther, fixed in acetic alcohol and stored at least for two days in 70% alcohol, after a thorough washing in water in a 1% solution of Clarase, a proprietary enzyme complex, for 10 minutes to several hours, according to the plant material. Apparently the enzyme complex Pectinol (43) can be used for the same purpose.

(ii) Zirkle (76) has developed ingenious combined fixing, staining and mounting media. They can be used with water-soluble and fat-soluble mounting media. A modified medium of the last type has also been recommended by Wilson (75). The new stains orcein and lacmoid can be used to replace carmine in all these solutions. In practice, Zirkle's methods are rapid and may serve some purposes; optically, however, the preparations are not always entirely satisfactory.

(iii) To prevent evaporation of the stain it is necessary to seal the edges of the cover slip. A most convenient method is to seal with one of Zirkle's water-soluble media (40). With carmine and Feulgen preparations Speese (60) has suggested the use of a modified Zirkle solution in which the aceto-carmine is replaced by 45% acetic acid, 80 cc. 45% acetic acid, 10 cc. Karo corn syrup (dextrose) and 10 cc. Certo (pectin). Where permanent slides are required, the medium readily dissolves in any of the acetic mixtures employed to separate the slide and cover slip.

(iv) Under tropical conditions acetic-stains deteriorate. Apart from refrigeration, the keeping qualities of carmine solutions are

improved by addition of Igepon T, an emulsifier and wetting agent (55).

(v) In the making of permanent preparations it is now most usual to employ Euparal as a mountant. It was first used in this connection in the vapour method for salivary glands (8). With Euparal or its American equivalent, Diaphane, the preparation can be mounted direct from 95% alcohol. Precipitation of the stain, which occurs with alcohol-xylol mixtures, is thus avoided. Both mountants can be used with orcein, but with lacmoid these neutral media change its colour to a less satisfactory brown. For carmine and orcein they can be used as follows:

Separate slide and coverslip by inverting in acetic alcohol (1:3); dehydrate in 95% alcohol two changes, 2–3 minutes in each. Mount rapidly to avoid clouding through humidity. In England, where the humidity is greater, it is best to mount from absolute alcohol.

Hillary (31), on the other hand, with carmine prefers mounting in dioxan balsam. Here the slide and cover slip are separated in 50% acetic acid. They are then both flooded with two changes of dioxan, 2 minutes in each, and recombined in dioxan balsam. Success with this method largely depends on adjusting staining to avoid coloration of the cytoplasm. Any excess will be precipitated to give less clear definition.

Somatic Squashes

Materials. Where roots are unobtainable any rapidly developing tissues, such as leaf buds, petals, tendrils or glumes, provide a valuable source of mitoses (1, 24). Satisfactory preparations of any of these can be obtained by methods applied to roots.

Pre-treatment. The orientation of metaphase spindles to the long axis of the root, at one time limited the application of squashes to plants with large and few chromosomes. This can now be overcome by pre-treatment with drugs to inhibit spindle formation. Metaphase plates then accumulate with super-contracted chromosomes. To avoid too much concentration, O'Mara (50) recommends soaking excised roots in 0.01% colchicine for 2–3 hours. Meyer (46), working with guayule, claims that paradichlorobenzene is more suitable for this material. A saturated solution in water is used and treatment extends from 1–4 hours. The same author (45) uses a 0.2% solution of colchicine for excised leaf tips exposed to light. Darlington and La Cour (21) find that the concentration

of colchicine can be as low as 0.05%, depending on the greenness of the leaves.

Cold treatment can also be used successfully to super-contract the chromosomes. For this purpose Hill and Myers (35) recommend a one-day treatment at 2° C. for grasses. Darlington and La Cour (22) found that transference of *Trillium grandiflorum* from 24° C. to 0° C. for 2 days also greatly raised the proportion of mitoses in metaphase. More recently Warmke (73) has found it possible to take excised roots and treat in water at 0° C. for 1½ hours before fixing in Benda fluid also cooled at 0° C.

Choice of method. Numerous methods for the preparation of somatic squashes exist. Undoubtedly those made by Feulgen methods are superior, particularly where the best preservation of all stages of the mitotic cycle are required. The choice of fixative is wide. Staining is clear and precise, but fading is considerable after two or three years.

With acetic-stain techniques the choice of fixative is limited. On the other hand, preparations are easily made and can generally be rendered permanent without much deterioration. Fading is negligible. In this class, perhaps the simplest method consists in the direct killing and staining of tissues in acetic-lacmoid containing a drop of N.HCl, followed by gentle heating. For example, satisfactory preparations of rye root tips can be made in this way in less than ten minutes. With most plant somatic tissues, however, a short fixation in acetic alcohol is desirable (20).

Suggested improvements. The following suggestions have been made for improving the quality of Feulgen and acetic squashes:

Hillary (31, 34) has introduced two new methods of making permanent Feulgen squashes. The simpler consists in shredding the stained root tips by sharp needles in water-corn-syrup. A coverslip is then put on and pressure applied to separate and flatten the cells. In the other method the stained roots are dehydrated in three changes of dioxan and then transferred to dioxan-balsam for shredding and mounting. A preliminary treatment is generally necessary to assist cell separation. After fixation and washing, the roots are immersed in 4% (by volume) NH_4OH at 60° C. for 15 minutes to remove the pectic salts of the middle lamella. The roots are then thoroughly washed prior to hydrolysis to remove the now soluble pectate. It is also claimed that with the removal of these bases staining is more satisfactory.

With a similar purpose in view, Fabergé (27) has shown that pectic salts can be removed by an active enzyme complex obtained from the stomach fluid of the edible snail, *Helix pomatia*; garden snails are suitable, but provide relatively small amounts (21). A 1% aqueous solution of a standardized enzyme product called Pectinol, obtained from *Aspergillus*, is used in a recent squash technique of McKay and Clarke (43). Their method, using propionocarmine for staining, is lengthy, judged by other squash standards.

Whitaker (74) finds Benda fluid (39) the best fixative for Feulgen squashes. The same worker has introduced Euparal in the making of permanent Feulgen preparations.

Darlington and La Cour (2) find with Feulgen root tip squashes that bleaching after fixatives containing osmic acid is not necessary except where it may inhibit staining of small chromosomes. Excessive blackening of the cytoplasm is removed almost entirely where squashing occurs immediately after staining.

Where chromosome structure is of importance, the same authors (17) recommend the use of fixatives low in acetic acid, to reduce bubble artifacts to a minimum.

For Feulgen squashes the period of fixation is best limited to a few hours. Otherwise excessive hardening by formalin or osmic acid in chromic type fixatives may interfere with squashing. Where materials must by necessity remain over a week-end, hardening can be minimised by introducing additional 1% chromic acid, in equal quantities with the fixative, to the vial containing the fixed tissues.

Warmke (72), working with *Melandrium* where the chromosomes are small, uses a section-Feulgen squash method. The root tips are fixed in a suitable chromic type fixative and sectioned transversely by the paraffin method. A modified Feulgen method is used, hydrolysis being extended to 45 minutes. After staining the sections are washed for 10 minutes in running water prior to the first sulphite wash. In the finished preparation which is mounted in thin balsam, the cells separate readily, allowing any cell to be flattened. In my opinion this method is the most satisfactory for plants with small chromosomes and where the roots may be fine and otherwise difficult to handle.

Burrell (9), with small chromosomes, finds that thinly cut transverse free-hand sections provide larger numbers of countable metaphase plates. The sections are fixed in aceto-carmine and afterwards

treated with Warmke's fluid (equal parts conc. HCl and 95% alcohol); then restained in carmine, mounted and squashed.

Meyer (46) recommends an acetic-orcein method after pre-treatment with paradichlorobenzene. Orcein was originally suggested by the present author (40) for use with root squashes. With most plants acetic-lacmoid is now preferred, partly because of the difficulty in obtaining suitable samples of orcein.

Rosen (54) has devised new rapid orcein techniques for root tips and leaves. Root tips after pre-treatment with colchicine are fixed in a mixture of 2 parts absolute alcohol, 1 part chloroform before squashing in acetic-orcein. For waxy leaf material a small amount of chloroform is introduced to the acetic-stain.

Schedule 2. *Permanent Acetic Lacmoid Squashes*

Tissues: All somatic tissues and embryo-sacs

1. Fix in acetic alcohol (1:3) 15 minutes to 24 hours, for some plant root tips, *e.g.*, cereals and grasses, it may be advantageous to omit.
2. Transfer the tissues to a watch-glass containing a few drops of 7:10 cc. acetic-lacmoid plus 1 cc. N.HCl.
3. Heat without boiling 2-5 times over a spirit flame; leave to stain 5-10 minutes; vary the amount of heating according to the hardness of the tissues.
4. Crush the tissues on the slide in a drop of fresh acetic-lacmoid. The flat end of a bone or metal needle holder is a good instrument for this purpose. With roots crush thin sections of the tip; for embryo-sacs omit crushing, except with the largest material.
5. Cover with an albumenized cover slip. Apply pressure under several thicknesses of blotting paper, avoiding sideways movement of the cover slip.
6. Heat gently 2-3 times over a spirit flame to aid contrast and stick the cells.
7. Separate slide and cover slip by inverting in a ridged dish containing acetic alcohol (1:3).
8. Rinse in absolute alcohol, 2 changes 1 to 2 minutes in each.
9. Recombine in thick cedarwood oil, thickened by exposure to air.

Notes: (i) In large ovule tissue where penetration of the stain may have failed, separate slide and cover slip (after step

- 6) in 45% acetic acid. Restain by flooding the squashed tissues with fresh acetic-lacmoid.
- (ii) With embryo-sacs to be followed by Feulgen staining (see Section 5), separate likewise in 45% acetic acid. Harden the tissues prior to hydrolysis in 70% alcohol overnight.

The Feulgen Anther Squash

The anthers, as with somatic tissues, are smeared after fixation and staining in bulk. In my opinion the method should be employed only where the anthers are too dry to be smeared or too difficult to be treated otherwise. In this connection Hillary (31, 34), with acetic alcohol fixation, has obtained good preparations of prophase stages from plants hitherto considered poor material. He was thus able to show chiasmata at diplotene in *Gasteria* where prophase is diffuse and difficult to interpret. Here again pre-treatment may prove a valuable asset. Swanson (67), by keeping flowering spikes in water in an incubator at 40° C. for 24 hours, was able to study in *Tradescantia* the prophase stages which are otherwise diffuse.

To secure rapid penetration it is usually necessary to fix the anthers or small pieces of large anthers in acetic alcohol. To avoid over-maceration of the tissues with hydrolysis, harden the tissues after a 1-hour fixation in 95% alcohol overnight. Where staining is poor or where the tissues are prone to damage by squashing after staining, it may prove more practical to achieve cell separation by an enzyme method. The cells can then be squashed before staining by teasing out in a drop of 45% acetic acid on an albumenized slide. To secure sticking of the cells, the slide is passed 2 or 3 times over a spirit flame. The tissues are then hardened by separating the slide and coverslip in 50% alcohol, where they should remain for 2 to 4 hours before proceeding with hydrolysis.

Anther squashes are valuable for pollen grain mitosis, particularly where the pollen grain walls are thick and persistent. Here pre-treatment in Warmke's HCl and alcohol mixture, used as a preliminary to hydrolysis, will assist in the escape of the cytoplasm. (See schedule 3.)

The Feulgen Embryo-sac Squash

Hillary (34) recommends Feulgen squashes for the preservation of meiosis in embryo-sacs. On the other hand, Darlington and La

Cour (20, 23) find it more profitable to squash by the lacmoid method (see Schedule 2) and then follow with Feulgen. Staining, particularly of early prophase, is then more efficient. The method also has the advantage that division stages can be determined in lacmoid, and unwanted slides discarded.

The best squashes in either method are obtained with ovules dissected either singly or in strings. Removal of unwanted tissues also aids penetration of the fixative and makes searching in the finished preparation easier.

Schedule 3. *The Feulgen Squash Method*

Tissues: All, except anthers which should as a rule be treated as smears

1. Fix 2-24 hours (anthers with acetic alcohol, 1 hour; harden in 95% alcohol overnight).
2. Wash in water 2-30 minutes; maximum time with fixatives containing formalin.
3. Bleach after osmic fixatives in 1 part 20 vol. H_2O_2 , 3 parts 80% alcohol, 3-6 hours.
4. Hydrolyse in N.HCl at 60° C. after aqueous fixation, 10-15 minutes; alcoholic fixation, 6 minutes.
5. Stain in leuco-basic fuchsin 2 hours.
6. Bleach in SO_2 water, 3 changes 10 minutes in each.
7. Tease out small pieces of tissue (roots, thin slices of the tip) in 45% acetic acid with the blunt end of the needle holder.
8. Cover with an albumenized cover slip and press by blotting, allowing no sideways movement of the cover slip.
9. Heat slide gently over a spirit flame 4 or 5 times; do not boil.
10. Separate slide and cover slip by inverting slide in a ridged dish containing 40% alcohol.
11. Pass the cover slip, and slide if necessary, through alcohols: 80% 2 minutes, absolute 2 changes, 2 minutes in each.
12. Recombine slide and cover slip by mounting in Euparal or Diaphane.

Notes: (i) After alcoholic fixation tissues can be transferred direct from 50% alcohol to the N.HCl.

(ii) Omit step 3 with root tips, except where the chromosomes are small.

- (iii) Step 6 can usually be avoided, except with pollen-mother-cells and in a specific test for desoxyribose nucleic acid.
- (iv) For pollen grains with thick walls. Treat after fixation in equal parts conc. HCl and 95% alcohol 10–15 minutes. Harden in Carnoy (6:3:1) 20 minutes.

Schedule 4. *Feulgen Section—Squash Method* (72)

Tissues: Roots, fine or with small chromosomes

1. Fix in suitable chromic acid type fixative 12–24 hours.
2. Wash, dehydrate and embed by the paraffin method (Warmke recommends dehydration in n-butyl alcohol and embedding in rubber-parawax).
3. Cut traverse sections (thickness depends on chromosome size).
4. Dry slides and remove the wax in xylol; pass through graded alcohols to water (bleach after osmic acid fixation in 1 part 20 vol. H_2O_2 , 3 parts 80% alcohol, 4–12 hours).
5. Wash slides in running water 30 minutes.
6. Hydrolyse in N.HCl at 60° C. 45 minutes.
7. Stain in leuco-basic fuchsin 2 hours.
8. Intensify by washing in running water 10 minutes.
9. Bleach out diffuse stain in 3 changes of SO_2 water, 3–10 minutes in each.
10. Pass up alcohol series to xylol.
11. Mount in thin xylol-balsam, using No. 1 cover slips; leave 3–15 days before squashing.
12. Locate a cell suitable for squashing and apply local pressure to one side of the section containing the cell. In this way, with the aid of a lower power objective, a selected cell is easily separated free of surrounding cells. Flattening is obtained by applying slight pressure directly over the isolated cell.

Schedule 5. *Feulgen-Light-Green Method* (58)

(1) *Sections*

Tissues: All. Fixatives: without acetic acid, or an aqueous fixative with acetic acid if treated before hydrolysis, in 1% chromic acid for 4–6 hours, and afterwards thoroughly rinsed in water and placed in 75% alcohol for 4 hours.

1. Cut and prepare sections by the paraffin method. Remove wax and pass down alcohols. Rinse in water; leave in 75% alcohol 2-3 hours.
2. Rinse in warm water, 2-3 changes; leave 5 minutes.
3. Hydrolyse in a vessel of 12% HCl at 60° C. 20 seconds.
4. Cool slides by immersing vessel in cold water.
5. Replace acid with cold N.HCl 1-2 seconds.
6. Stain in leuco-basic fuchsin 2 hours.
7. Bleach in SO₂ water, 3 changes 10 minutes each.
8. Rinse in distilled water, in 50% alcohol, then in 70% alcohol.
9. Mordant in 80% alcohol saturated with Na₂CO₃ 1 hour.
10. Rinse in 80% and 95% alcohol 1 second each.
11. Stain in a filtered saturated alcoholic solution of light green, to which is added 2 or 3 drops of pure anilin oil, 20-25 minutes.
12. Drain off excess dye, rinse in a saturated solution of Na₂CO₃ in 80% alcohol, 10 cc.; 80% alcohol, 90 cc.
13. Differentiate in 95% alcohol till green remains only in the nucleoli.
14. Dehydrate in absolute alcohol 2-3 changes, alcohol-xylol (1:1), alcohol-xylol (1:3).
15. Xylol, mount in neutral balsam.

(2) *Squashes*

Follow procedure in Schedule 3 to step 10. Rinse in 70% alcohol, then proceed as above from step 9.

(3) *Smears*

Follow procedure in Schedule 1 to step 11. Rinse in 70% alcohol, then proceed as above from step 9.

THE PARAFFIN METHOD

Dehydration and Infiltration

In the United States of America, at least, the old ethyl alcohol-xylol or ethyl alcohol-chloroform techniques have largely been replaced by speedier methods employing new reagents. In general, they are claimed, besides shortening the procedure, to reduce hardening and shrinkage to a minimum. Among such reagents introduced some ten years ago (39), the most important are dioxan, *n*-butyl alcohol and tertiary butyl alcohol. Their application to the paraffin technique has been the subject of further review (36, 44, 47).

In England, however, most workers, including the present writer, find the ethyl alcohol-chloroform method (39) preferable to all others. No difficulty is experienced in securing infiltration of the wax, and what is most important, the bulk of it is done at a relatively low temperature, a method less inductive to shrinkage than any other (cf. 64). The hardening of tissues is negligible for plant chromosome work.

Paraffin Technique

In recent years some valuable information pertaining to the paraffin method has been provided (20, 36, 44). The second author gives photographs of paraffin ribbons illustrating some faults common in section cutting.

Randolph (53) has devised a card mount method for the handling of large numbers of root tips or similar tissues. Fresh tissues are attached to card mounts with any adhesive which hardens rapidly in aqueous fixation and is insoluble in the lower alcohols. After fixation and dehydration to 70% alcohol the tissues are transferred to permanent cards on which they are orientated for sectioning. A glue which hardens rapidly in 80% alcohol and is insoluble in infiltrating media is used in making the permanent cards. In practice, where large amounts of material are to be handled, the saving in time and of dehydrating reagents is said to be considerable.

STAINS FOR CHROMOSOMES

Apart from stains used in combined stain-fixatives, the principal stains are leuco-basic fuchsin and crystal violet; the former is of wider application. The leuco-basic fuchsin method was originally developed (28) as a microchemical test for desoxyribose-nucleic acid found in chromosomes.

It gives to the chromosomes in all tissues, whether treated in bulk or as a single layer of cells, an intense and fairly permanent coloration. The colour depends on the fixative used; violet after alcoholic fixatives, red after chromic. The cause of earlier failures with the reaction can now be attributed to one of the following:

(i) Hillary (33) has shown that maximum reaction depends on the correct timing of hydrolysis. With fixatives containing chromic acid, maximum staining follows hydrolysis at 60° C. from 6-30 minutes; without chromic acid it is limited to 4-8 minutes.

(ii) The same worker (33) has also shown that osmic acid, unless

reduced by bleaching, may interfere with staining. In Feulgen root squashes this is serious only where the chromosomes are small.

(iii) With Navashin fixatives and the like, the reaction ceases to be specific, unless all the formalin is thoroughly removed by washing.

(iv) Poor samples of basic fuchsin, which fail to give completely decolorized solutions, may give diffuse and indistinct staining. This happens less frequently nowadays with the standardisation of stains. As a precaution, however, the solution is best made up as advised by Coleman (13). Recently Rafalko (52) claims better staining with solutions bleached by direct charging of SO_2 gas.

(v) Finally, from the observations made by various workers (21, 68) it is shown that the nucleic acid charge on the chromosomes may vary with temperature and other conditions. Thus because of its specificity for desoxyribose-nucleic acid, the Feulgen reaction is sensitive, as a stain, to changes in environmental conditions.

The crystal violet method (39) is simple and rapid. It is possible to stain sections up to $40\ \mu$ thick with well-stained chromosomes and clear cytoplasm. Unlike the Feulgen method, however, it needs careful differentiation to give the best results. This is particularly true with small chromosomes, where the stain may be too easily removed. In such cases the slides can be treated with a 1% aqueous solution of chromic acid either as a mordant before staining (30, 70) or as a precipitant after staining (39). For the latter purpose picric acid (36, 70) can be substituted with success.

A more satisfactory method may prove to be the one suggested by Semmens (56), wherein differentiation is practically avoided. A mild hydrolysis in N.HCl at 60°C. , as a preliminary to staining, presumably removes other stainable fractions, *i.e.* ribose-nucleic acid. The slides are stained in a 1% aqueous crystal violet solution for 1 hour; then rinsed in water and rapidly passed through 75%, 95% and absolute alcohols into xylol. The same author (56) has also introduced a new method of making up the dye solution, which is said to give superior staining with greater permanency. In my own experience, using two samples of crystal violet, no benefit was to be found over ordinary methods of preparation. Nevertheless the method may prove useful where poor samples of dye are experienced.

In recent years haematoxylin has seldom been used in any serious plant cytological work, but where a similar kind of staining is re-

quired, it can be obtained more easily with chlorazol black E. This new dye was first suggested for use as a nuclear stain by Cannon (10) and further recommended by Conn (16). When used as a 1% aqueous solution for 1-2 hours, little or no differentiation is required.

NUCLEOLI AND THE FEULGEN STAIN

Nucleoli are Feulgen-negative; some form of counter-staining is therefore necessary with this stain where a study of nucleoli is to be made. A simple fast green method for slides or bulk tissues to be squashed (0.1% aqueous solution for 2-12 hours) has been recommended (34) to be used after the SO_2 bleach and rinsing. An orange filter can be used to provide additional contrast. For the study of abnormal nucleoli behaviour more specific staining may be desirable. The light green method (4, 57, 58) is then the best. (See Schedule 5.)

TECHNIQUE FOR POLLEN TUBE MITOSIS

Artificial germination of the pollen is necessary for study of this mitosis. For this purpose cane sugar is employed as follows:

- (1) As a 3%-30% solution in water.
- (2) The same in agar (3) or in 2% agar, 2% gelatin (49).

The sugar percentage will vary with the species and the individual; the correct percentage must therefore be determined by experiment. A rough guide to sugar concentrations has been provided by Darlington and La Cour (21).

In Newcomer's method (49) the pollen is sown on a slide thinly smeared with agar-gelatin media. Germinated by keeping in a moist chamber; killed in Navashin fluid; stained with crystal violet.

Introduction of the floating cellophane method (41) provides, perhaps, the most favourable means of germinating pollen. It also allows the use of any fixing and staining method, including the Feulgen reaction (see Schedule 6).

The method can be combined with mitosis-arresting agents where division does not happen too rapidly. With successful treatment the chromosomes lie in single file along the length of the tube (66).

Schedule 6. *Floating Cellophane Method for Pollen* (41)

Uses: Pollen germination tests and pollen tube mitosis

1. Wet cellophane square (about 2×2 cm.) in sugar solution. Remove excess by blotting.

2. Float square on a large drop of sugar solution in a Petri dish. Keep upper surface free of solution.
3. Sow ripe pollen by dusting on the square. Replace Petri dish lid. Keep in a temperature of approximately 20° C.
4. Examine periodically under the microscope.

For germination studies, staining is not generally necessary. The following methods are suitable for study of pollen-tube mitosis:

(i) Fix and stain in acetic lacmoid 5 minutes by covering the square face upwards with the solution on a slide.

(ii) Fix in acetic alcohol 2-24 hours. Stain in leuco-basic fuchsin after 6 minutes hydrolysis at 60° C.

Permanent preparations can be made with both methods. With lacmoid, rinse rapidly in acetic alcohol (1:3) then proceed as from step 8 in Schedule 2. After Feulgen, rinse in 45% acetic acid, then proceed as from step 11 in Schedule 3.

The cellophane must be not thicker than 0.04 mm. and of the non-waterproof type. To accumulate metaphases 0.05% colchicine can be combined with the sugar solution or acenaphthene crystals can be scattered in the Petri dish (66).

SPECIAL TREATMENTS

Pre-treatment for Structure

Where chromosomes are large, their spiral structure is sometimes favourably seen at meiosis in acetic-stain smears. Success depends on immediate killing of the cells; where it is hindered by several cell layers or thick walls, *e.g.*, pollen grains, the chromosomes may appear as vacuolated structures (*cf.* 14). A more certain means of showing spiral structure is to loosen the coils by pre-treatment. Of the methods devised for this purpose, the most important employ ammonia fumes, ammonia in alcohol, acid fumes, desiccation or NaCN, NaHCO₃, NaOH and other weak alkaline salts in aqueous solutions.

The first four (39) are satisfactory only where the chromosomes are large. Weak alkali solutions (14, 15, 38), however, are of wider application; they give greater detailed structure, with large and small chromosomes alike. As to the method, Coleman (14) recommends treatment of smears in a NaCN 2⁻⁵ mol. solution for 15 seconds to 3 minutes, according to the plant material. To avoid loss of cells the solution is gently flooded over the smear and removed with equal care by filter paper.

In a survey of the most useful fixatives to employ after pre-treatment, Coleman and Hillary (15) find that acetic alcohol (in a 1:2 solution) is best. After fixation the smears can be stained either with an acetic-stain or by the Fuelgen reaction. With the last, to avoid losing cells during hydrolysis, the slides to carry the smears must be quite dry and scrupulously clean.

Hillary (34) finds that pre-treatment, when followed by partial digestion of the cell proteins, allows the structure to be more favourably seen. In practice, however, the method is not easy; it is extremely difficult to maintain the cells on the slide.

As yet no method of wide application is available for demonstrating spiral structure in somatic chromosomes. In certain circumstances it may be seen by the following means:

(i) Pollen grain, first mitosis (25, 29). Accident in fixation with acetic-stain, followed with pressure on the cover slip to separate the coils.

(ii) Pollen tube mitosis (71). Extremely thin walls permitting easy access of the stain-fixative.

(iii) Pre-meiotic mitosis (14). Anther smear pre-treated in weak alkali solution ($\text{NaCN}2^{-5}$ mol.).

(iv) Root tips in plants with heterochromatin (18, 19). Plants grown at 0°C . for 3-4 days to give nucleic acid starvation of the heterochromatin segments.

(v) Root tip mitosis (4). Roots pre-treated in mercuric nitrate solution (0.005 mol.) or solutions of other heavy metals. To give with crystal violet staining, differential staining of the centric and telomere regions in chromosomes at metaphase and anaphase.

Technique for Revealing Heterochromatin

Low temperatures restrict the supply of nucleic acid to the prophase chromosomes. With suitable cold treatment, heterochromatin can be made visible because of its lower control in its nucleic acid attachment (17-19). To be successful the treatment must extend for the greater part of the resting stage. In root tips the nucleic acid starvation of the heterochromatin may be seen after a few hours treatment, but a 3- or 4-day treatment gives greater numbers of starved metaphases. With longer treatment spindle formation may be inhibited, or cell division stopped. For the same reason the temperature at which the treated plants are grown should,

as a rule, never be lower than 0°C . With some plants it may be as high as 6°C .

With pollen grains the length of treatment will vary with the length of the resting stage. It may be as long as 180 days (*i.e.*, *Trillium grandiflorum*), although work with *Fritillaria pudica* (19) indicates that the temperature can be increased for short intervals during the course of treatment. Indeed, with most plants intervals of higher temperatures may be necessary to secure pollen grain division.

As yet, little information is available regarding the treatment of meiosis in plants. My experiments (unpublished) indicate that in plants treatment can not usually be successful without affecting chromosome pairing. Starved heterochromatin can, however, be shown in *Rhoeo discolor* at the first metaphase after a six-day treatment at 6°C . Here, even at this temperature, chromosome pairing is affected to give a larger proportion of cells with complete rings of the twelve chromosomes. Starvation can be shown at the second meiotic division where a resting stage occurs between the two divisions. Thus with *Fritillaria lanceolata* a one-day treatment at this stage of development is successful.

X-rays affect nuclear nucleic acid metabolism. Because of this they sometimes render visible intercalary heterochromatin segments by starvation. X-rays can be combined with cold treatment to show the relative effects of irradiation on euchromatin and heterochromatin (22).

Apparently in certain circumstances heterochromatin can be seen at metaphase in root tips by careful differentiation in the crystal violet technique (70).

Histo-Chemical Tests

With advance in our knowledge of cell chemistry, cytologists have come to depend on methods of a histo-chemical nature to provide confirmatory and indisputable evidence on certain cell aspects. These are mostly in connection with the two nucleic acids found in cells:

(a) Desoxyribose-nucleic acid is usually attached to the protein thread of the chromosomes. Its presence can be confirmed by the Feulgen reaction, despite criticism (11, 12, 62, 63), provided certain necessary precautions are taken in its use (2, 28, 61, 65, 68).

(b) Ribose-nucleic acid is found in the cytoplasm and nucleoli, and in heterochromatin in association with desoxyribose-nucleic acid (7, 68). It is Feulgen-negative. A method devised by Brachet (6) can be used for detection and even for its estimation in the cell constituents (7, 51). Its validity depends upon the use of a ribonuclease in combination with Unna's methyl-green-pyronin stain. Cell constituents stain pink to red, according to the concentration of ribose-nucleic acid; the chromatin green. In the nuclease-treated control the chromosomes alone should be stained. Osmic acid in the fixative inhibits the Unna double-staining reaction; the chromatin then stains red-violet, leaving the nucleolus and cytoplasm unstained. Acid hydrolysed tissues behave similarly. Tissues should, therefore, be fixed in either acetic alcohol or Zenker formol (Helly's fluid) the latter most certainly where the tissues are to be sectioned. Squashes can be used for Brachet's test if an enzyme method is used for cell separation (21).

Protein histo-chemical tests are perhaps of little value to the ordinary cytologist. Of the methods available, the simple arginine reaction introduced by Serra (59) may be the most useful. The reaction is not differential, but the chromosomes react more strongly than the cytoplasm.

Schedule 7. *Methyl-Green-Pyronin Method (Unna Pappenheim)*
(Modified for ribose-nucleic acid discrimination; (6, 7, 51)

Tissues: All. Fixatives: alcoholic or Zenker formol

Smears or sections, use two slides or two halves of one slide for test and control.

1. Rinse in distilled water.
2. Transfer test slide to distilled water in which a small amount of crystalline ribo-nuclease is dissolved; keep at 50° C. 2-3 hours.
3. Stain test and control together in methyl-green-pyronin, 20-30 minutes.
4. Rinse in distilled water.
5. Drain and dry by careful blotting.
6. Dehydrate in absolute alcohol 1 part: acetone 1 part: xylol 6 parts; 10 minutes.
7. Xylol, mount in balsam.

Crystalline ribonuclease is difficult to obtain. A method of preparation is given by Kunitz (37).

FORMULAE

(additional to La Cour 39)

Acetic-lacmoid. (Recommended English dye source, British Drug Houses.) Solubility differs with dye source. The stain as an indicator is known as "lacmoid", as a dye "resorcin blue". The standard solution is 1% in 45% acetic acid. Because of deterioration in dilute acid after about 14 days, the stain is best kept as a 2.2% stock solution in glacial acetic acid which can be diluted to 45% as required. Thus dissolve 2.2 gm. lacmoid in 100 cc. glacial acetic acid by gentle boiling. Cool, dilute and filter as required.

Acetic-orcein. (Recommended English dye source, G. T. Gurr, London.) The standard solution is 1% in 45% acetic acid, prepared from stock as for lacmoid. American synthetic orceins need greater dye concentration.

Leuco-basic fuchsin. (Modified formula after De Tomasi; cf. 13, 39). Dissolve 1 gm. basic fuchsin by pouring over it 200 cc. boiling distilled water.

Shake well and cool to 50° C.

Filter; add 30 cc. N.HCl to filtrate.

Add 3 gm. $K_2S_2O_5$.

Allow solution to bleach for 24-48 hours in a tight-stoppered bottle in the dark; add 0.5 gm. decolorizing vegetable carbon.

Shake well for about a minute and filter rapidly through coarse filter paper.

Store in tightly stoppered bottle in the dark.

SO_2 water (use freshly prepared).

5 cc. N.HCl.

5 cc. $K_2S_2O_5$ 10%.

100 cc. distilled water.

Methyl-green pyronin (Unna Pappenheim).

0.25 gm. phenol	}	Solution A.
100 cc. distilled water		
1 gm. methyl green		

0.25 gm. phenol	}	Solution B.
100 cc. distilled water		
1 gm. pyronin G		

For use:—3 parts A: 7 parts B.

Crystal Violet (56). Shake up 10 gm. of crystal violet with 100 cc. of ethyl alcohol.

Filter; discard the precipitate; concentrate the filtrate by gentle heat to about 10 cc.

Then leave to dry completely at room temperature.

For use, dissolve 1 gm. of the dried extract in 100 cc. of distilled water.

Stain-fixative mounting media (76).

No. 1.

10 gm. gelatin.

10 cc. sorbitol.

50 cc. glacial acetic acid.

60 cc. distilled water.

0.5 gm. $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$.

Carmine to saturation.

No. 2.

20 cc. Venetian turpentine.

55 cc. phenol (as loose crystals or 88% liquid).

35 cc. propionic acid.

10 or 15 cc. glacial acetic acid.

25 cc. distilled water.

0.5 gm. $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$.

Carmine to saturation.

Mix in a graduated cylinder in the following order: propionic acid, turpentine mix thoroughly; phenol, acetic, water. The ferric nitrate should be dissolved before any carmine is added.

Filter after 12 hours.

At no time should the mixture be heated.

Do not allow fluid to come into contact with the skin.

Note: Orcein or lacmoid can replace the carmine in both of the above media. The iron salt is then unnecessary.

Lewitsky's Fluid.

10% formalin

1% chromic acid.

Mix in equal proportions fresh for use.

Zenker Formol (Helly's fluid).

5 gm. corrosive sublimate

2 gm. potassium bichromate

1 gm. sodium sulphate.

100 cc. distilled water.

For use, to 20 cc. of the above add 1 cc. formalin.

Semmens Fluid.

10 gm. chromic acid.

100 cc. distilled water.

When dissolved add 20 gm. sodium diuranate.

For use, dilute 1 part with 9 parts of water.

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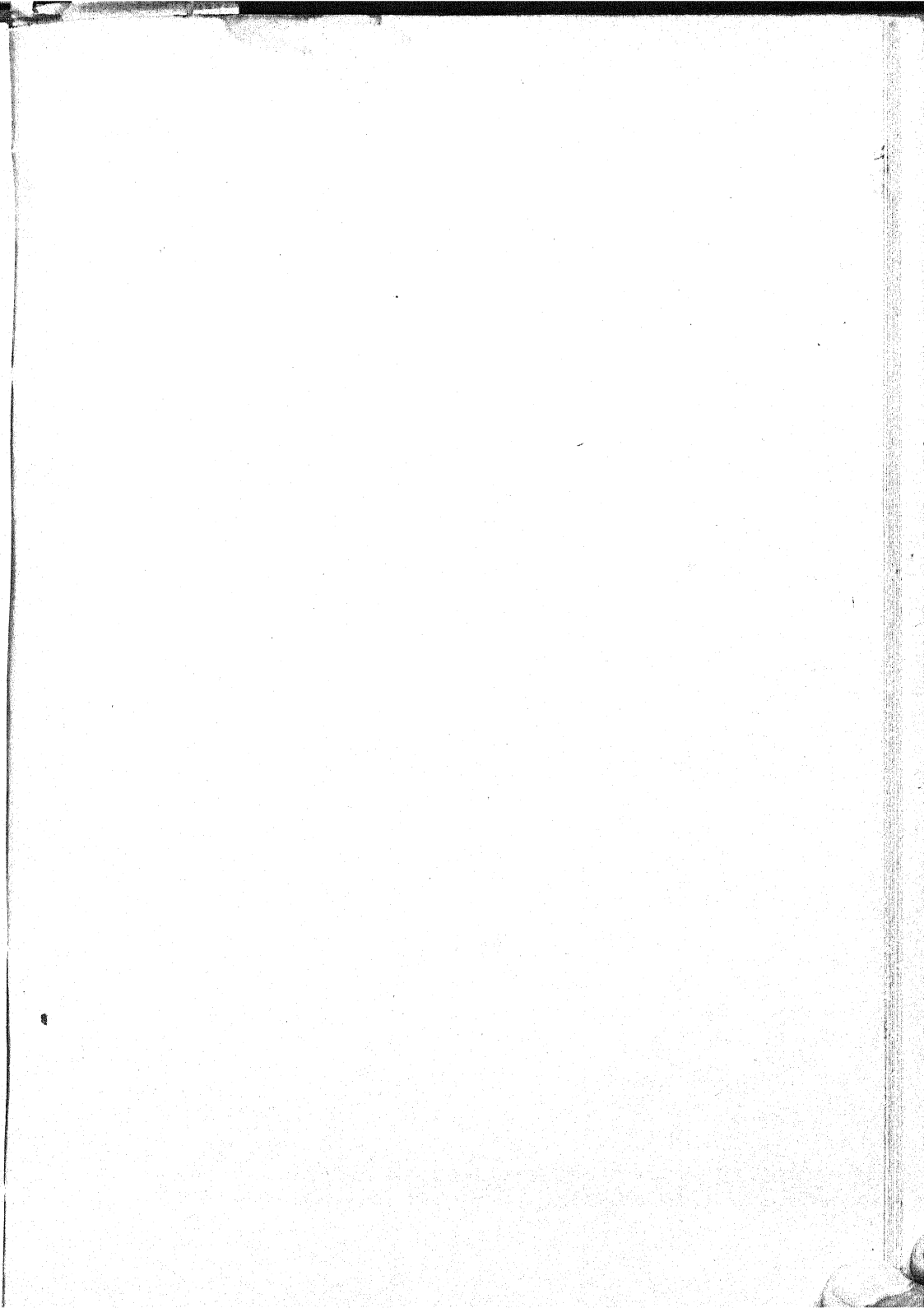
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THE ANTHOCYANIN PIGMENTS OF PLANTS

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Zurich, Switzerland*

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INTRODUCTION

Owing to their optical properties, the anthocyanins belong to the most prominent of secondary plant substances. For this reason they have always aroused the interest of botanists and chemists.

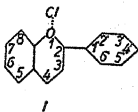
The continuation, by P. Karrer and R. Robinson, of the fundamental chemical investigations carried out by Richard Willstätter, has led to a far-reaching clarification of the chemistry of these pigments. Only the constitution of the nitrogenous anthocyanins and that of the leuco-anthocyanins is in need of further investigation.

Unfortunately, the situation with regard to these plant pigments is not so favourable when looked at from the botanical point of view. It is true that a large number of observations on the behaviour of anthocyanins in plants are recorded in the literature, but as yet no complete picture can be obtained on the basis of the results of these investigations concerning the physiology and significance of the anthocyanins.

This review, comprizing chiefly the research work of the last two decades, *i.e.*, since the appearance of the second edition of Onslow's "The Anthocyanin Pigments of Plants"—hopes, therefore, to stimulate investigators to further research on this "vegetable chameleon", as it has been called by the gifted botanist Tswett (538).

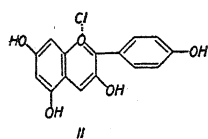
CHEMISTRY AND BIOCHEMISTRY

The anthocyanins, usually dissolved in the cell sap, may be found chiefly in flowers and fruits, though also in other plant organs. According to the fundamental investigations of Willstätter and his collaborators (557-577), these pigments belong to a group of glycosides, the sugar-free pigments or aglycons of which are called anthocyanidins. The various anthocyanidins are derivatives of 2-phenylbenzopyrylium, given here in the form of the chloride (I) and as such also designated as flavylium chloride:

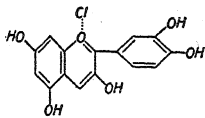


Anthocyanidins

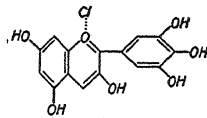
Investigations (213, 447, 448, 451, 575) have shown that the following anthocyanidin types, represented as chlorides, may be distinguished:



II
Pelargonidin
(3,5,7,4'-tetrahydroxy-
2-phenylbenzopyry-
lium chloride)

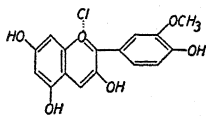


III
Cyanidin
(3,5,7,3',4'-penta-
hydroxy-2-phenyl-
benzopyrylium
chloride)

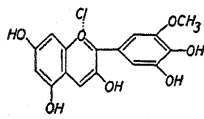


IV
Delphinidin
(3,5,7,3',4',5'-hexa-
hydroxy-2-phenyl-
benzopyrylium
chloride)

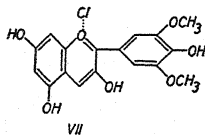
From these three anthocyanidins the following methoxylated anthocyanidins may be derived:



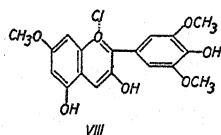
V
Peonidin
(3,5,7,4'-tetrahydroxy-3'-
methoxy-2-phenylben-
zopyrylium chloride)



VI
Petunidin
(3,5,7,4',5'-penta-
hydroxy-3-methoxy-2-phenylben-
zopyrylium chloride)

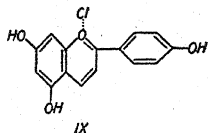


VII
Malvidin
(3,5,7,4'-tetrahydroxy-3',5'-
dimethoxy-2-benzopyr-
ylium chloride)



VIII
Hirsutidin
(3,5,4'-trihydroxy-7,3',5'-
trimethoxy-2-benzo-
pyrylium chloride)

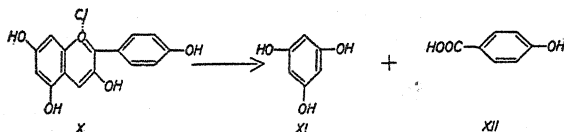
As a special anthocyanidin the following may be mentioned:



IX
Gesneridin
(5,7,4'-trihydroxy-2-phenylbenzopyrylium chloride)

Proof of the constitutional formula for the anthocyanidins, apart from being based on the conversion of flavonols into anthocyanidins, which will be dealt with at length later, is founded on the results obtained from fusion with potassium hydroxide (559). By treating the anthocyanidins in this manner, two simple products are obtained,

a phenol and a substituted phenolcarboic acid. Pelargonidin chloride (X), for example, is decomposed into phloroglucinol (XI) and p-hydroxybenzoic acid (XII):



This method of degradation is not particularly well adapted to methoxylated anthocyanidins, since the methoxyl groups are also saponified during the fusion with potassium hydroxide. In such cases the method of degradation in which dilute barium or sodium hydroxide (10%) in an atmosphere of hydrogen is used (222), gives good results. It makes possible exact allocation of the methoxyl groups in the anthocyanidin molecule. With certain anthocyanidins, degradative oxidation with hydrogen peroxide (227) can also be used.

Anthocyanins

Anthocyanidins have been observed in plants only in rare cases (206, 207, 209, 459, 500, 562). As a rule, they occur in nature attached to one or more sugars, as anthocyanins.

By boiling the anthocyanins for a short time in 20% hydrochloric acid, the pigment may be split into the anthocyanidin and the sugar components (575). Oxidative degradation with hydrogen peroxide (224, 227) results in certain anthocyanins in oxidation products which are still closely allied to the original anthocyanins.

The anthocyanins appearing in nature are partly mono-, partly di-glycosides. Until now, glucose, rhamnose, galactose and gentiobiose have been isolated as sugar components. One of these sugar molecules is always attached at the 3-position. If a second sugar molecule is present, it is either coupled with the first or attached to the anthocyanidin in the 5-position.

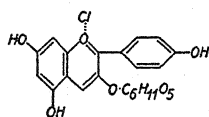
The majority of anthocyanins appearing in nature may be grouped as follows (448):

- a) 3-monoglucosides and 3-monogalactosides
- b) 3-rhamnoglucosides and other 3-pentoseglycosides
- c) 3-biosides

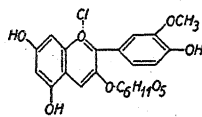
d) 3,5-diglucosides

e) acylated anthocyanins

Callistephin (XIII), one of the pigments from the aster, a 3-monoglucoside of pelargonidin, oxycoccicyanin (XIV), a peonidin 3-monoglucoside from the skins of *Oxycoccus macrocarpus*, and fragarin, the related galactoside from the strawberry, are representatives of the first group:

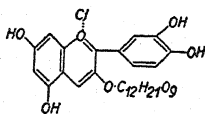


XIII
Callistephin

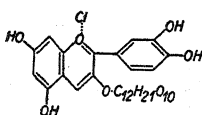


XIV
Oxycoccicyanin

Keracyanin (XV), cyanidin 3-rhamnoglucoside, from black cherries, and mecocyanin (XVI), cyanidin 3-gentiobioside, belong to Group 2 and 3:

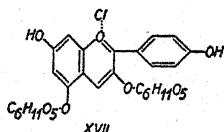


XV
Keracyanin

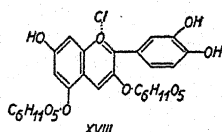


XVI
Mecocyanin

The 3,5-diglucosides are the most widely distributed in nature; they are also the best known. Pelargonin (XVII) from *Pelargonium zonale* and cyanin (XVIII) from *Centaurea cyanus* have already become known (557, 560):



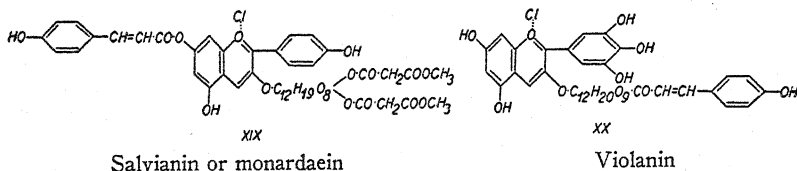
XVII
Pelargonin



XVIII
Cyanin

A number of plants (*Salvia splendens*, *Viola tricolor*, *Gentiana acaulis*, etc.) contain anthocyanins in ester combination with an organic acid (68, 216, 223, 226, 279, 286, 567). So far, malonic, p-hydroxybenzoic, p-hydroxycinnamic and 4-hydroxy-, 3,5-dimethoxycinnamic acid have been obtained from the degradation of these acylated anthocyanins. The acid radicals either can be in

ester combination with one of the hydroxyl groups of the anthocyanidin (245) or can be attached to an hydroxyl group of a sugar component. The first and second possibilities seem to apply to salvianin or monardaecin (XIX), obtained from *Salvia splendens* and *Monarda didyma* (226); the latter variant is true of violanin (XX), obtained from the flower of *Viola tricolor* (216):



Isolation and Properties of Anthocyanins and of Anthocyanidins

All anthocyanins are soluble in water, as is shown by the fact that they are present in the cell sap of vacuoles; the anthocyanidins, on the other hand, are not soluble in water. The glycosides are insoluble in non-hydroxylic solvents, such as ether, chloroform and benzene.

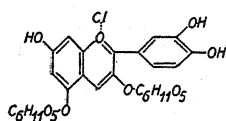
Molisch (343) discovered the important fact that anthocyanins in solution produce well crystallizing products when treated with acids. This fact was used (575) in the isolation and purification of these pigments. Subsequently, the anthocyanins were shown to be amphoteric substances which build oxonium salts with acids.

As a rule, anthocyanins are extracted from well desiccated plant material by means of methyl alcohol containing 1%–2% hydrochloric acid. The crude anthocyanin chloride is precipitated with a threefold volume of ether. The precipitated chloride is then re-dissolved and once again precipitated by means of ether. This purifying process is repeated, according to necessity. The final recrystallization is then carried out *via* the picrate or chloride.

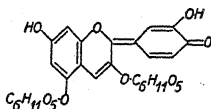
Anthocyanins usually appear as mixtures in plants. The components of these mixtures may be separated either by fractional crystallization of the picrates (222) or by use of the chromatographic adsorption technique (219, 221).

In solution, the oxonium salts of anthocyanins are red in colour; nevertheless, the individual types can easily be distinguished by the shade of colour. Cyanin, for example, is red in solutions of pH 3.0 or less, violet at pH 8.5 and blue at pH 11.0. The red form of

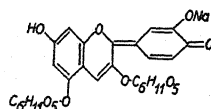
cyanin is the oxonium salt (XXI). The violet form of cyanin is represented by the colour-base (XXII), to which a quinoid structure may be attributed (45, 46); this latter thesis is supported also by others (37, 198, 455). The conception that the blue and violet forms of anthocyanins have a quinone-similar structure, is supported, apart from chemical considerations, by the fact that all anthocyanins and anthocyanidins appearing in nature show an unclosed hydroxyl group in the 4'-position. The blue form of the cyanin is present in the salt of the colour-base (XXIII):



Oxonium salt



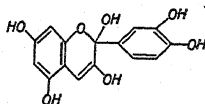
Colour base



Na salt of the Colour base

Some investigators (142, 329, 396) have made use of these properties of the anthocyanins, utilizing them as indicators, whereas others (433) have investigated the behaviour of the pigments in buffered solutions.

Numerous anthocyanins and anthocyanidins change into a colourless modification, the pseudobase, in very weakly acid, neutral and especially alkaline solution. In the last the oxonium salt can be regenerated by use of strong acids. This pseudobase (XXIV) of the cyanidin may be considered in all probability to have the following structural formula (220, 274, 559):



Pseudobase

In aqueous and alcoholic solution the anthocyanins and anthocyanidins, which possess two neighbouring phenolic hydroxyl groups, show a colour change towards violet and blue with ferric chloride. This sensitive reaction is not present with other anthocyanins and anthocyanidins (213). Robinson and Léon (453) have made use of the resistance of very dilute pigment solutions to ferric chloride in order to ascertain whether position 3 of the

anthocyanins or of the anthocyanidins has an hydroxyl group or not.

Anthocyanins show increased blueness with increase in the hydroxyl groups and change from the 3- to the 3,5-sugar type. Methylation of one or more hydroxyl groups, on the other hand, increases the redness of these pigments.

Some of the important properties of anthocyanidins are summarized in the table on page 249 (213).

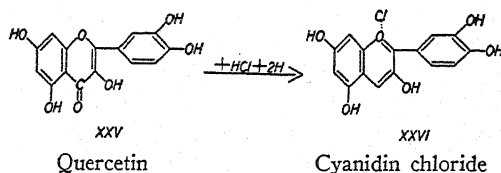
Absorption Spectra of Anthocyanins and Anthocyanidins

Anthocyanins and anthocyanidins have approximately the same absorption spectra (481). These compounds absorb very strongly in the investigated range of 6,000 Å–2,000 Å, and an absorption maximum is present in the visible range. The anthocyanins and anthocyanidins investigated by Schou (pelargonidin, cyanidin, delphinidin, peonidin, syringidin, malvin) all show a band at about 2,700 Å.

Further investigations on the spectra of the flavylium salts have been carried out (170–174, 529). Among other things, the relation between light-absorption and hydroxyl- and sugar-substitution were more closely investigated in these researches. The pigment of red cabbage in various solvents has been spectroscopically investigated (383, 384).

Syntheses of Anthocyanidins and Anthocyanins

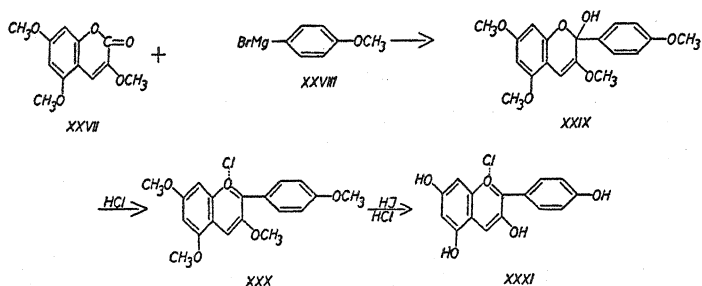
The first synthesis was that of cyanidin chloride (XXVI) (577). Quercetin (XXV) was reduced with magnesium in aqueous methyl alcoholic hydrochloric solution to cyanidin chloride (XXVI). The yield, however, was poor:



A short time afterwards synthesis of pelargonidin chloride (XXXI) was achieved by addition of p-anisylmagnesium bromide (XXVIII) to 3,5,7-trimethoxy-coumarin (XXVII), in the following manner (580):

SOME IMPORTANT PROPERTIES OF ANTHOCYANIDINS

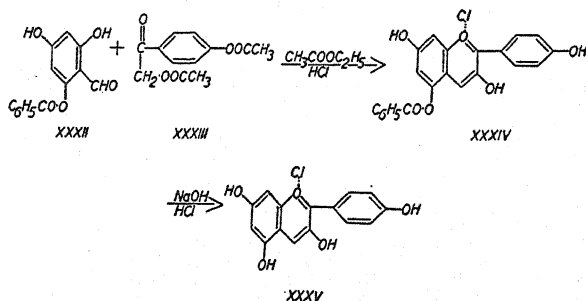
	PELARGONIDIN	CYANIDIN	DELPHINIDIN	PEONIDIN	MALVIDIN	HIRSUTIDIN
COLOUR OF AQUEOUS SOLUTION	Red	Violet red	Bluish red	Violet red	Violet red	Violet red
SOLUBILITY OF THE CHLORIDE IN WATER	Readily soluble	Only slightly soluble in dilute hydrochloric acid	Very soluble	Readily soluble	Slightly soluble	Slightly soluble
FERRIC CHLORIDE REACTION	Not definite	Intense blue	Intense blue	Not definite, only faint	No reaction	No reaction
BEHAVIOUR TOWARDS FEHLING'S SOLUTION	Reduces when warmed	Reduces in the cold	Reduces in the cold	Reduces when boiled	Reduces when boiled	Reduces when boiled
BEHAVIOUR IN AQUEOUS SOLUTION	Colour fades on standing	Colour disappears on heating (Isomerisation)	Slow fading in the cold; when heated rapid fading (Isomerisation)	Colour disappears on heating	Colour disappears in very dilute solution when heated	Colour disappears in very dilute solution when heated
COLOUR CHANGE IN SODA SOLUTION	Blue	Violet, then blue	Violet, then blue	Violet, then blue	Violet, then greenish blue	Violet, then greenish blue



In similar fashion cyanidin chloride and other anthocyanidins have been synthesized (578, 581).

Robinson and his co-workers approached the problem of the synthesis of anthocyanidins and anthocyanins from an entirely different angle (450), by using condensation of ortho-hydroxybenzaldehydes with appropriate ketones followed by ring closure. This procedure can be traced back to Perkin, Robinson and Turner (376), but has been developed by R. Robinson to a greater degree of perfection, so as to afford a general method for the synthesis of almost all anthocyanidins and anthocyanins.

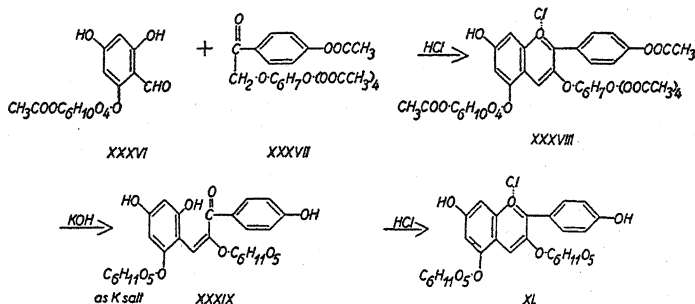
As an example, synthesis of pelargonidin chloride (XXXV) (435) may be cited. The condensation of O-benzoylphloroglucinaldehyde (XXXII) and ω :4-diacetoxyacetophenone (XXXIII) in ethyl acetate solution in the presence of hydrogen chloride leads to the formation of 3:7:4'-triethoxy-5-benzoyloxyflavylium chloride (XXXIV), which on debenzoylation is changed to pelargonidin chloride (XXXV):



A whole series of compounds of the anthocyanidin type, including those which are present in nature as well as those which have

not yet been isolated, have been synthesized in a similar manner (37, 38, 58, 59, 125, 181, 198, 322, 355, 368, 388, 389, 390, 391, 392, 393, 394, 395, 430, 431, 432, 435).

Pelargonin, the anthocyanin in petals of *Pelargonium zonale*, *Centaurea cyanus*, *Dahlia varibilis*, etc., has been synthesized in the following way from 2-O-monoacetyl- β -glucosidylphloroglucinaldehyde (XXXVI) and ω -O-tetraacetyl- β -glucosidoxy-4-acetoxyacetophenone (XXXVII) (457):



Many other anthocyanins have likewise been synthesized (25, 26, 107, 138, 139, 141, 300, 301, 302, 304, 305, 306, 319, 354, 357, 420, 421, 426, 427, 428, 429, 434, 445, 455, 456, 457). By means of these syntheses the structural formulae for many anthocyanins isolated from plants have been established beyond all doubt.

Further researches on syntheses have been carried out as well (246, 247, 248, 251).

Chemical Relationship of Anthocyanidins to Other Classes of Plant Products

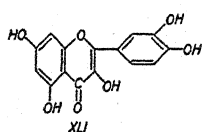
As early as 1914 Willstätter and Mallison (577) were able to demonstrate the intimate relation between anthocyanidins and other plant substances. These two workers succeeded in transforming the flavonol, quercetin (XLI), to cyanidin chloride (XLII) by means of reduction with magnesium in aqueous methyl alcoholic hydrochloric solution. This reduction was at the same time the first partial synthesis of an anthocyanidin. In the same manner other flavonols occurring in nature were successfully changed to flavylum salts (12, 13, 14, 250, 252). Quercetin was reduced to cyanidin chloride by means of titan trichloride (228).

Freudenberg and co-workers (111) were successful in reducing

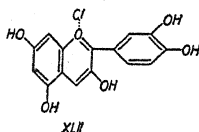
cyanidin chloride (XLII) to d,1-epicatechin (XLIII) by catalytic hydrogenation.

The reaction in the reverse direction was first carried out by Appel and Robinson (9). By the action of bromine in hot technical dioxan solution on d-catechin tetramethyl ether, they obtained a bromocyanidin tetramethyl ether bromide, which gave cyanidin chloride upon demethylation. Chater (60) was able to carry out this reaction successfully on a quebracho extract. Direct oxidation of the catechin to cyanidin chloride was recently achieved with various oxidizing agents (290).

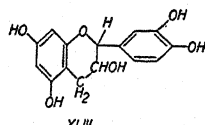
If the anthocyanidins, for example, cyanidin (XLII), are compared with the flavonols, for example, quercetin (XLI), and with the catechins, for example, d,1-epicatechin (XLIII), the anthocyanidins lie between the flavonols and catechins from the point of view of degree of oxidation:



Quercetin (Flavonol)



Cyanidin (Anthocyanidin)



d,1-Epicatechin
(Catechin)

Factors Affecting the Colours of Anthocyanin Pigments in Plants

The various colours of tissues containing anthocyanins are due not only to the various anthocyanins themselves but also to changing amounts and mixtures of them, to alterations in the pH of the cell sap, to the variable ash content of the latter, to co-pigmentation and to the colloidal condition of certain other components of the sap. Willstätter and Mallison (566) were the first to recognize these factors clearly.

Simultaneous presence of several anthocyanins. The foregoing authors (566) were able to isolate a mixture, composed chiefly of cyanin and only a little pelargonin, from a violet-red variety of *Pelargonium*. By means of capillary analysis, Tsakalotos (537) was able to recognize two anthocyanins in the pigment of *Vaccinium Myrtillus*. Karrer and Widmer (222) later succeeded in separating this pigment into its component parts by means of fractional crystallization of the picrates. In further investigations, Karrer and co-workers (219, 221) separated mixtures of pigments by means of

chromatographic absorption technique as well. The diversity of the pigment mixtures occurring in nature is also made manifest by the numerous researches of Robinson and collaborators (23, 296, 297, 400, 440, 441, 442, 443).

Variability of anthocyanin content. To what extent the colour of flowers is determined by the quantity of anthocyanins present has likewise been made clear (566). The cornflower, for example, is very poor in pigment; the flower petals contain only 0.65%–0.70% cyanin, calculated on dry weight. On the other hand, a dark purple variety contained 13%–14% pigment. *Pelargonium peltatum* contained 1%, *Pelargonium zonale* 6%–14% pelargonin in the dry petals.

G. M. Robinson (438) has also observed variations in the anthocyanin content of flowers; the results of her researches will be dealt with in greater detail later.

Hydrogen-ion concentration of cell sap. Willstätter and Everest (557) already recognized the significance of the reaction of cell sap for alteration of colour. Nevertheless, exact measurements were undertaken only much later. In this connection it was realized (51–53, 448) that blue varieties possess a higher pH than red ones; nevertheless, these differences are often too small to explain the different colours. These results have been verified to a large degree (379). Scott-Moncrieff (490) was able to demonstrate the influence of the cell sap reaction on the colour of flowers in extensive experiments. She ascertained—as did R. and G. M. Robinson—the reaction of freshly ground petals by means of a glass electrode. The values determined lay almost without exception on the acid side of the neutral point. The differences in pH between red and blue petals in related plants usually amounted to only 0.5–1 pH unit; they were smaller than those obtained by another method (379). Karrer and Schwarz (218), on the other hand, were unable to detect any relationship between the colour of flowers and their content of steam-volatile organic acids.

Despite the properties of anthocyanins as indicators, a much more important rôle than that accorded to the usually over-estimated cell sap reaction is played by other factors in the colour changes of anthocyanins in plant tissues (co-pigmentation, colloidal condition).

Ash content of the cell sap. The influence of the ash content of cell sap on the determination of colour is a question which has not

yet been completely clarified. Karrer and co-workers (227) have determined the ash content of various red and blue flower petals. Blue flowers contained more than red ones. This evidence is regarded as a proof that the colour of red flowers is determined by oxonium salts, whereas that of blue flowers is determined by alkaline salts or by those of the alkaline earths of the anthocyanins.

Mihailescu (334) carried out similar researches with a large number of different but intimately related plants whose petals were coloured red or blue by anthocyanins. He claims, however, to have noticed no definite differences in the ash content of red and blue flower petals. In a further investigation this author determined the alkalinity of the ash contents and found a larger amount of alkaline substances in the ash of blue flower petals than in that of red petals (335).

These factors have been more successfully investigated in *Hydrangea*. Thus Storck (519) found that the juice obtained from blue flowers has a somewhat higher content of ash than that from red ones. The quantities of iron present in red and blue flowers were equal, whereas the quantity of aluminium in blue flowers definitely surpassed that of iron. Analyses of pink and blue flowers of *Hydrangea* also indicate the great significance of aluminium in the blue ones (63). Results in agreement with these were obtained by others (5, 79, 536, 553).

The researches of Robinson and collaborators have thrown an entirely new light on colour change in the flowers of *Hydrangea*. According to them (296) the pigment of the red, violet and blue flowers is delphinidin-3-monoglucoside, as could be ascertained also by comparison with the synthesized product obtained by Reynolds and Robinson (420). Using this synthetic anthocyanin, all colours of the various *Hydrangea* flowers were reproduced on filter paper with the aid of organic acids and tannin (454). The idea that ferric salts cause a blue colour can be thrown aside, physical conditions being paramount. Ferric salts, introduced into the ground as fertilizer, are said to bring about physiological disturbances resulting in reduction of the anthocyanin concentration, which, as will be described later, plays an important rôle in the determination of colour. Is it not possible, however, that the ferric salts also influence the colloids in the cell sap?

Co-pigmentation. Willstätter and Zollinger (572) long ago ob-

served that addition of tannin to a solution of oenin chloride in dilute hydrochloric acid intensified the colour and resulted in a much bluer red. Jonesco (206, 207) likewise pointed out the simultaneous presence of anthocyanins and tannins and their resultant additive complexes. Similar observations were made by Currey in investigations on rose petals (82). He found that the cause of blueing in red roses is insufficient tannin in the cell sap of the petals and that tannin is capable of forming an oxonium salt with the anthocyanin pigments.

This phenomenon, however, was investigated in detail for the first time by G. M. and R. Robinson (440) and was designated as co-pigmentation. Further researches on the interaction of flavones and anthocyanins were carried out by Lawrence (294). So far, the known pigments (co-pigments) occurring in nature are anthoxanthins (flavones and flavonols) and tannins. Nevertheless, *in vitro* other substances can attain the blueing effect of the natural anthocyanins in dilute, strongly acid solution. This phenomenon is not the result of salt formation; it is evidently the result of formation of weak additive complexes, and varies according to the co-pigment and anthocyanin concerned.

A characteristic example of this phenomenon is shown upon close investigation of the flower of *Fuchsia*. The violet-colored inner corolla contains tannin and for this reason may be distinguished from the outer red petals.

The most widely distributed co-pigment is the flavonol quercetin which has recently been isolated together with a nitrogenous anthocyanin from the bracts of *Bougainvillea glabra* (399). Kaempferol, found in the flowers of *Crocus asturicus* and *Crocus speciosus*, etc., in great quantity (398), as well as butein obtained from *Dahlia variabilis* (397) and other widely-distributed anthoxanthins and tannins (150, 548), all influence, when simultaneously present, the colour of anthocyanins.

Colloidal condition. R. and G. M. Robinson (448) observed that the cell sap of the cornflower colours blue litmus red and even has a more acid reaction than the cell sap of the red rose. Both flowers contain cyanin. The cyanin must therefore be present in a complex form and not in the form of the potassium salt, as assumed by Willstätter and Everest (557). In point of fact, further investigation showed that a lyophilic colloid was present in the cell sap.

Willstätter and Everest as well (557) had already found xylan and other polysaccharides in their extracts of cornflowers. Jonesco (205), too, noticed the peculiar behaviour of cornflower extracts. The Robinsons suggest that cyanin in the blue cornflower cell sap is blue because it is absorbed on colloidal particles which stabilize the anions at a pH of 4.9.

Since deviations in pH are usually insufficient to explain alterations in colour of anthocyanin-containing tissues, the condition of the pigment in solution becomes extraordinarily important together with that of co-pigmentation. It does not seem excluded that all blue flowers may be coloured by colloidal solutions of their respective anthocyanins.

Nitrogenous Anthocyanins

A nitrogenous anthocyanin was isolated for the first time by Schudel (484). This was betanin, the pigment of the red beet, which upon analysis showed itself to have a nitrogen content. Later this very sensitive pigment was investigated by others (1, 403, 477) who also worked out a quantitative method of determination (404). Unfortunately the structural formula of betanin has not yet been determined, though some students (2, 436, 437) have undertaken synthetic researches in this direction.

Price and Robinson (399) were likewise able to isolate a nitrogenous anthocyanin, with an absorption spectrum similar to that of betanin, from the purple bracts of *Bougainvillea glabra*.

Nitrogenous anthocyanins have been observed (265, 297, 401) in the following plant families: Cactaceae, Nyctaginaceae, Onagraceae, Amaranthaceae, Basellaceae, Chenopodiaceae, Phytolaccaceae, Ficoideae, Papaveraceae and Portulacaceae. In his investigations on plant pigments on the Galapagos, Taylor (530) found that a high proportion of the plants contained nitrogenous anthocyanins.

Leuco-anthocyanins

Rosenheim (459) isolated a white amorphous powder of glycosidic nature from the unripe berries of purple and white grapes. He was able to demonstrate that treatment with hydrochloric acid in the absence of oxygen resulted in production of an anthocyanidin. Although Rosenheim had no idea of the nature of this compound, he called it "leuco-anthocyanin".

Subsequently, Jonesco investigated the properties and distribu-

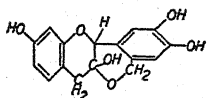
tion of the leuco-anthocyanins (209). However, his researches on the leuco-anthocyanins in the seedling of *Fagopyrum esculentum* have been disputed (229).

In this hitherto relatively untouched field, G. M. and R. Robinson (439, 442, 443, 444) have once again carried on important pioneering work. They divide the leuco-anthocyanins into three classes:

- (a) those that are insoluble in water and the usual organic solvents, or which give only colloidal solutions;
- (b) those readily soluble in water and which can not be extracted from the solution by means of ethyl acetate;
- (c) those capable of extraction from aqueous solution by means of ethyl acetate.

Class (b) probably consists of relatively simple glycosides or diglycosides, whereas members of class (c) are sugar-free and should be regarded as leuco-anthocyanidins.

The heartwood of *Peltogyne porphyrocardia* is light-brown upon sectioning; in the air and in light it becomes characteristically purplish-red. The Robinsons (444) were able to isolate in crystalline form a representative of class (c) from this wood. On the basis of chemical and physical investigations, the following formula (XLIV) is attributed to this compound, called peltogynol:



XLIV

Formation of an artificial anthocyanidin, peltogynidin, from peltogynol, seems to involve oxidation, but the authors were not able to inhibit the appearance of pigment on boiling with hydrochloric acid by taking ordinary measures for the exclusion of oxygen.

As a rule, the anthocyanidins originating from the leuco-anthocyanins are identified only by a system of qualitative tests and comparisons in solutions. G. M. Robinson (439), however, also succeeded in transforming a substance contained in the gum of *Butea frondosa* into crystalline cyanidin chloride. The latter was then analysed, and upon comparison with an authentic specimen, proved to be genuine cyanidin chloride. Cyanidin has not been formed from the gum by the action of hydrochloric acid alone; preliminary

oxidation is necessary, and under special conditions use of picric acid has been found to be advantageous.

According to some investigations (297, 442, 443), leuco-anthocyanins are present in wood, bark, nutshells, flowers, leaves and fruits. They are morphologically much more widespread than the normal anthocyanins. In Criollo cacao, for example, leuco-anthocyanins are present in practically all parts of the seed and fruit (240); Cornforth and Earl (77) found leuco-anthocyanins in the fruit of *Pittosporum undulatum*.

The anthocyanidins resulting from boiling leuco-anthocyanins with hydrochloric acid that have hitherto been tested, have proved to be cyanidin in 84% of the cases. As in the flavones and flavonols, the anthocyanidins chiefly possess the dihydroxy-grouping (130), a proof or indication that the cyanidin structure is the one most readily produced in the plant.

When anthocyanins and leuco-anthocyanins are both present in the same part of the plant, the normal anthocyanin is not in every case (*Hydrangea opuloides*, *Vitis heterophylla*) derived from the same anthocyanidin as the leuco-anthocyanin. There are also cases where mixtures of anthocyanidins were obtained from leuco-anthocyanins, analogous to those found with normal anthocyanins (bark of *Dipterocarpus zeylanica*, wood of *Eucalyptus tereticornis*, etc.).

OCCURRENCE OF ANTHOCYANINS

Distribution throughout the Vegetable Kingdom

For a long time anthocyanins were isolated exclusively from flowering plants, but without a doubt they are also present in other representatives of the vegetable kingdom.

Data published on them in bacteria (7, 44) are regarded as erroneous (95). After testing with chemical methods, the latter authors were unable to verify the observations (496) claiming an anthocyanin in *Bacillus Citrulli Sartoryi* and in *Actinomyces violaceus ruber* Waksman (263).

Möbius (340) is not of the opinion that the red cell sap of *Ancylonema Nordenskjöldii* contains anthocyanin; likewise, researches of long standing have shown that there is no anthocyanin in the alga *Mesotaenium*. The reports (148) that anthocyanins are present in these algae do not therefore seem to be based on

flawless evidence and do not sound very convincing. Thus, no anthocyanins have been so far detected in micro-organisms.

Anthocyanins are certainly in mosses (183), and the claim of their presence in ferns (126) has been verified in *Azolla*, *Adiantum*, *Osmunda*, etc. (23, 349, 401, 530).

From all these observations it is very apparent that anthocyanins are present in the whole of the plant realm from the mosses upwards; a cyanidin 3-monoside even in Coniferae (23).

Systematic Distribution of the Anthocyanins

The Robinsons and their collaborators have developed a number of qualitative tests—based on the chemical behaviour of anthocyanins and anthocyanidins prepared synthetically or isolated from natural sources (440, 441, 442, 443). With the aid of these the anthocyanins present in crude plant extracts can be typified. These tests are based on colour reactions with alkalis and ferric chloride (433), distributions between immiscible solvents (305, 484) and the so-called "oxidation" test. In the last case a dilute solution of the pigment is shaken with 10% aqueous sodium hydroxide in the presence of air.

Using these tests an exhaustive survey was made by these authors on the occurrence of anthocyanins and anthocyanidins in the vegetable kingdom. Apart from the flowers, other anthocyanin-containing organs of the plants were also investigated (23, 296, 297, 400, 440, 441, 442, 443). Schmid and Körperth (476) likewise investigated a series of flower extracts by means of this procedure.

Anthocyanins and anthocyanidins have been isolated from the following plants, and their constitution has been ascertained in a number of cases:

<i>Abrus precatorius</i> L. (seed-coat) (129)	<i>f. rubra</i> (L.) (leaves) (66, 68, 69, 555, 556)
<i>Acer circumbolatum</i> Max and A.	<i>Centaurea cyanus</i> (flower) (557)
<i>ornatum</i> Carr. (coloured autumn leaves) (169)	<i>Chrysanthemum indicum</i> L. (flower) (458, 568)
<i>Althaea</i> sp. (flower) (563, 221, 222)	<i>Commelina communis</i> var. <i>hortensis</i> Makino (flower) (277, 278, 279)
<i>Ampelopsis quinquefolia</i> Michx. (fruit) (572, 222)	<i>Cyclamen persicum</i> Mill. (flower) (222)
<i>Antirrhinum majus</i> L. (flower) (486)	<i>Delphinium consolida</i> L. (flower) (561)
<i>Aster chinensis</i> L. (flower) (458, 569)	<i>Fatsia japonica</i> Decaisne et Plan- chon (fruit) (178)
<i>Brassica oleracea</i> L. var. <i>capitata</i> L.	

- Gaillardia bicolor* Hook (flower) (568)
Gentiana acaulis (flower) (223)
Gesnera fulgens (flower) (445)
Gladiolus sp. (flower) (568)
Glycine Soja Benth. (seed-coat) (280, 282, 284, 286)
Helenium autumnale L. (flower) (568)
Hibiscus Sabdariffa L. (flower) (589, 590)
Hyacinthus orientalis (flower) (175)
Ipomoea hederacea (flower) (588)
Iris ensata Thunberg var. *hortensis* Makino et Nemoto (flower) (179, 180)
Lycoris radiata Herb. (flower) (176)
Malva silvestris L. (flower) (222, 564)
Monarda didyma (flower) (223, 225)
Nicotiana Tabacum (flower) (587)
Olea Europaea L. (fruit) (356)
Oxycoccus macrocarpus Pers. (fruit) (140)
Paeonia spec. (flower) (177, 219, 222, 565)
Papaver Rhoeas L. (flower) (473, 474, 475, 570, 574)
Pelargonium zonale (flower) (560)
Perilla ocimoides L. var. *crispa* Benth. (leaf) (249, 283, 286)
Petunia hybrida hort. (flower) (573)
Pharbitis Nil Choisy. (flower) (230, 231, 232)
Pirus Malus (fruit) (91, 464)
Primula hirsuta (flower) (224)
Primula integrifolia (flower) (224)
Primula polyanthus (flower) (487)
Primula viscosa (flower) (224)
Prunus avium L. (fruit) (570)
Prunus spinosa L. (fruit) (570)
Punica granatum (flower) (223)
Ribes rubrum L. (fruit) (568)
Rosa gallica (flower) (558)
Rubus sp. (fruit) (217)
Saccharum officinarum (rind) (331, 411, 461, 540)
Salvia patens Sello & S. *coccinea* L. (flower) (226, 567)
Sambucus nigra L. (fruit) (223, 366, 367)
Solanum melongena L. var. *esculentum* Ness. (fruit) (281, 285, 286)
Solanum tuberosum (tuber) (67)
Sorghum sp. (coleoptile) (410)
Tropaeolum majus L. (flower) (568)
Tulipa Gesneriana L. (flower) (568)
Vaccinium Myrtillus (fruit) (222, 562, 572)
Vaccinium Vitis idaea (fruit) (559)
Verbena hybrida (flower) (493)
Vicia sp. (flower) (223)
Viola tricolor (flower) (216, 571)
Vitis sp. (fruit) (8, 43, 76, 222, 373, 459, 500, 562, 572)
Zea Mays (seed) (465)
Zinnia elegans (flower) (568)

Anthocyanin as a Character of Classification

The formation and presence of anthocyanins as a character of classification is of importance for the division of many cultivated plants. Gassner and Straib (123) were able to observe that in rye and wheat, in general, the intensity of the red colouring of the coleoptiles varies, depending on the particular kind. Anthocyanin formation can not always be observed in barley and oats. For this reason it is recommended to study anthocyanin formation in the lower leaf sheaths for purposes of classification. In all kinds of grain the coloration of coleoptiles and leaf sheaths is not an absolute indication; it is dependent to a great extent on external factors (temperature, light, ground conditions, etc.)

Clark, Martin and Ball (71) have utilized anthocyanin formation for the classification of American kinds of grain, Newman (361) for Canadian varieties. Voss (542) has even made use of this

characteristic in order to differentiate the various *Triticum* species, and later for classification of the glumes of *Tr. vulgare* (543).

Aufhammer (15) is of the opinion that anthocyanin formation can be used to differentiate summer and winter barley; others (132, 238) draw attention to the fact that cultivators of barley for brewing purposes should pay more attention to the formation of pigment.

In potatoes Snell (508) has found a relation between the colour of germinating seed and that of the flower. Plaut (382) and Snell (509) found similar relations between the coloration of fully-grown beets (hypocotyl and root) and that of the other plant organs.

Priebs (402) makes use of the colour of germinating seed to classify cabbages. In lettuce anthocyanin formation is also an important character of classification. Bos (34) has more closely investigated the conditions of anthocyanin formation and suggests making use of the capacity for anthocyanin formation in itself as a character of classification.

According to Sprecher (512), Criollo cacao has no coloured cotyledons, whereas the cotyledons of Forastero cacao are clearly distinguished by anthocyanin. Wellensiek has pointed out the value of this selective characteristic for purposes of selection (551).

Jones (201) has investigated the distribution of anthocyanins in Japanese, Korean and Chinese varieties of rice.

According to Mohammad and Alam (342), the content in anthocyanins serves as a characteristic of classification also in *Sesamum indicum*, as it does in cotton (191). Funck and v. Rathlef (119) are of the opinion that a quantitative determination of anthocyanin would be of value for selective purposes in roses; Reinhold (417) shares the same opinion for red cabbage.

Geographical Distribution of the Various Anthocyanins

According to some researches (23), pelargonidin derivatives predominate in the flowers of tropical and subtropical species. Delphinidin derivatives are the commonest in temperate and alpine plants. Red-flowered forms have a greater survival value than blue in most tropical plants.

It is to Taylor (530) that we are indebted for important researches concerning the distribution of anthocyanin pigments on the Galapagos. A high proportion of the plants was found to contain nitrogenous anthocyanins.

MORPHOLOGY

Anthocyanins may be found in all organs of the plant from root to flower. This varied distribution of them renders it difficult to form a unified hypothesis, not only from the morphological but from the cytological point of view as well, as these pigments can be localized both in plasma and membrane as well as in the vacuoles. Nevertheless, exact consideration of the cytological and morphological data can be of great service in helping to explain their physiological functions.

Cytology

Plasma. Although in the majority of observed cases anthocyanins are present in solution in the cell sap, crystallized anthocyanin has occasionally been detected in the cell plasma. With the aid of such crystals Schorr (480) was able to follow the plasma streaming in *Allium Cepa*. The question as to how such anthocyanin crystals manage to reach the plasma remains unsolved.

Membrane. Anthocyanins are much more commonly stored in the cell wall in both the lower and higher plants. We are indebted to Herzfelder (183) for detailed researches on membrane coloration in mosses. She found these pigments in the cell walls of *Sphagnum*, *Marchantia*, *Preissia* and other plants. Molisch (349) found anthocyanins in the membranes of the leaf stems and roots of *Eichhornia crassipes*. Detailed investigations on the anthocyanins in the exines of pollen of various plants have been carried out (33, 479).

Vacuoles. A very peculiar phenomenon is presented by the formation of so-called anthocyanophores. They have been found in varieties of *Erythrea* (310), in the red berries of *Fuchsia* (515), in the flowers of *Iris germanica* and *Dianthus caryophyllus* (144, 145), in the perianth leaves of *Delphinium cultorum* (471) and in the coronary tubes of *Pulmonaria officinalis* and *P. rubra* (547). Their presence has further been observed in *Iris Reichenbachii* (17) and in red cabbage (99, 116).

Guillermond (143, 145) is of the opinion that the basic substance of the anthocyanophores is composed of tannins or mucous substances and pectins. He was able to observe exactly that the basic substance of the anthocyanophores is colourless (144). Nevertheless, the exact chemical composition still requires further investigation. The same remark applies to the blue granular ac-

cumulations obtained by application of molybden in tissue that contained anthocyanin pigments (546).

Küster (269) believes that formation of anthocyanophores may be the result of phenomena of mixture separation. On the basis of his own researches, Hofmeister (186) attributes to the anthocyanin pigments in the flower petals of Borraginoideae the capacity of aiding in excretion of solid particles from the cell sap.

The presence of crystallized anthocyanins in the juice of the blood orange has also been described (328).

Some (148) continue to hold the opinion that formation of anthocyanins in the plant cell has some relation to the chondriosomes. Küster (269), on the other hand, together with other French botanists, is of the opinion that these pigments are produced in the vacuoles.

Histology

In a comprehensive and fundamental investigation, Gertz (126) has carried out researches on the topographical distribution of the anthocyanin pigments in many families of the vegetable kingdom. Unfortunately, owing to linguistic difficulties, the results of his investigations are available only with difficulty. Even to-day his researches are a veritable treasure trove for anyone intending to work on the histology and physiology of the anthocyanins.

More recent investigations on the topography of the anthocyanins contain valuable directives which can help to increase our understanding for the physiological significance of these pigments.

Histochemical determination of the presence of anthocyanins. Histochemical determination of the presence of anthocyanins in solution can fairly easily be carried out (344, 539). Tissues containing anthocyanins are coloured red in the presence of acid vapours, whereas in the presence of ammonia vapour the colour change is through violet to blue. In an alkaline environment tissue sections very often become green; this is caused by a mixed colour resulting from blue anthocyanin and yellow flavone derivatives.

When tissues having cells containing anthocyanins are dipped into a yellowish nicotine solution, the cells become blue, violet or green.

When alcohol (50%), lead acetate (1%) or aluminium salt solutions are used, one obtains red or, mostly, violet or blue colorations and precipitates.

Under certain conditions one can bring about the crystallization of the oxonium salts from tissues rich in anthocyanins under the cover-glass. For this purpose the petals of *Pelargonium zonale*, *Dahlia* and *Rosa*, among others, may be used with a good deal of success. According to Molisch (343), the tissue is covered with acetic or hydrochloric acid, and the latter is then allowed to evaporate very slowly under cover of a bell jar. Red needles, needle clusters or conglomerates of the oxonium salt are then formed at the edge of the cover-glass.

Root and hypocotyl. According to Molisch, red root tips are present in a series of families (346): Crassulaceae, Saxifragaceae, Balsamineae, Melastomaceae and Compositae. The anthocyanin cells are either limited to the vegetation point or may still be found in the root cap. The same author (349) has observed in India the rather striking presence of anthocyanins in the roots of *Eichhornia crassipes*. In this case the blue pigment is limited exclusively to the cellular walls of the epidermis and the subepidermal cortex layers.

Whereas all cells of the hypocotyl and root of *Beta vulgaris* var. *Rapa* f. *rubra* usually are coloured, the red coloration of the hypocotyls of *Raphanus sativus* var. *radicula* is caused only by the anthocyanins in the subepidermal layer. In the hypocotyl of *Brassica oleracea* var. *capitata* f. *rubra*, as well, the anthocyanin pigments are limited to the subepidermal layer (116). The anthocyanin pigments in the hypocotyl of *Fagopyrum esculentum* are localized in the epidermis, the subepidermal layer and a few cells of the cortex (229).

Stem. According to Funk (120), *Monotropa hypopitys* var. *sanguinea* Hausskn. has a completely coloured stem. The epidermis and cortex are only slightly coloured. The greatest quantity of anthocyanin is stored in the phloem and the parenchymatous cells surrounding it.

Leaf. According to the distribution of the anthocyanin pigments, the following types may be distinguished (340):

- a). Green leaves with red stems and veins. The pigments are localized in the epidermis.
- b). The lower side of the leaf is red. In most cases it is the epidermis alone which is coloured, as in *Tradescantia discolor* and *Cissus discolor*; the pigments may, however, be in

- the hypodermis and in the spongy parenchymatous tissue, intensifying the colour there (*Vitis Henryana*).
- c). Green leaves with the upper side red. Owing to storage of anthocyanin pigments in parts of the epidermis, the leaves are mottled (*Orchis latifolia*, *O. maculata*).
 - d). Leaves of the blood variety. *Fagus silvatica* var. *atropurpurea* has its pigments only in the epidermis. Owing to this the arrangement may be mistaken for a coloured vascular system. Similar observations can also be made on red cabbage (116), although in this case the anthocyanins are localized in the hypodermal layer. Keener (236) has found the same distribution of anthocyanin pigments in the leaves of *Diervilla lonicera*. Other blood varieties show a completely coloured leaf tissue (*Aeschynanthus atropurpureus*, etc.)
 - e). The leaves become red upon opening. Distribution of the anthocyanin pigments varies greatly.
 - f). Red autumn leaves. In these it is mostly the epidermis or the parenchyma which is coloured.

Flower. The topography of the anthocyanins in the flower petals of *Anagallis arvensis* and *A. caerulea* has been investigated in detail (327). Only the outer cells of the periphery were coloured in a variety of *Myosotis* (64). Further information concerning the distribution of anthocyanin pigments in the petals can be found elsewhere (27, 72, 268, 292, 320, 478, 535).

The last author (535) has paid particular attention to the appearance of the various flower petal pigments in the angiosperms. In this investigation, which he extended throughout many families, he obtained extremely interesting results. He ascertained the following distribution of certain types of pigments, summarized here:

	YELLOW-WHITE GROUP	RED-BLUE GROUP
Choripetalae	80.7%	16.9%
Sympetalae	32.3%	60.3%
Compositae	89.1%	10.9%

Unfortunately, space does not permit us to treat in detail the conclusions concerning the morphological consideration of the petals, which Troll drew from these results.

A striking localization of anthocyanins has been described in the guard cells of the stomata of the fruit leaves of *Hyoscyamus niger* (102).

Anthocyanin pigments have been observed in the pollen of *Lythrum Salicaria* and in that of other plants (33, 479). They have also been noted in the anthers of *Petunia* (101) and the epidermis of the anthers of *Tulipa Gesneriana* and *Borrago officinalis* (340). Gertz (127) describes an interesting distribution of them in the flowers of *Daucus Carota* L.

Fruit. According to the investigations of Tobler (532), the pigment formed during maturation in the fruits of *Elais guineensis* later disappears. Dahlgren (83) reports similar behaviour in the outer coat of the fruits of *Viburnum lantanum*. The epidermis of the fruits of *Gunnera chilensis* contains anthocyanin pigments (347), the subepidermal layer of the fruits of *Decaisnea Fargesii* has them in crystalline form (515), and they develop in the aleuron grains of certain Gramineae during maturation (61, 62).

PHYSIOLOGY

Biogenesis of the Anthocyanins

From flavone-similar substances. Formation of anthocyanins from flavonols and flavone-similar substances is still supported by a number of authors, though others (50, 229, 298, 415, 452) look upon such a biogenesis as very improbable.

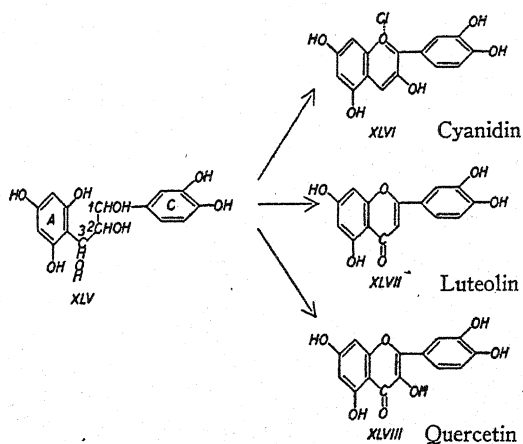
Guillermond (143, 145) assumes, on the basis of cytological investigations, that anthocyanins in petals are formed from oxyflavonols. Chaze (61, 62), too, believes that he can support this assumption on the basis of his researches on aleuron grains in the Gramineae. According to Störmer and von Witsch (518), it is a flavonol which is the precursor of the anthocyanin in *Petunia*. Rutzler (460) is of the opinion that flavones can be the precursors of autumn colours in various plants.

As did Willstätter and Mallison (577), who succeeded in transforming quercetin *in vitro* into cyanidin chloride by means of reduction, Noack (365) and Kuijper (275) also believed that anthocyanins are formed in plants by hydrogenation of flavonols. The former mentions formation of anthocyanins in the flowers of *Victoria regia* and *Cobaea scandens*, the latter in *Hibiscus mutabilis*, as examples. The same manner of formation seems very probable also to others (463-465), owing to the simultaneous presence of flavonol-glycosides and of the corresponding anthocyanins, both of which were isolated. Bancroft and Rutzler (18) hold to the opinion

that the red pigments of sumach, dogwood and barberry are formed by reduction of flavones.

In any case, the hypothesis that anthocyanins are formed in plants by oxidation from flavone-similar substances, which was already maintained in 1912 (e.g., 234, 235), still has its adherents to-day. Mascré (327) and Jonesco (208, 210) support this hypothesis on the basis of their investigations on the petals of *Anagallis* as well as on the red leaves of *Ampelopsis hederacea*, *Polygonum*, *Fagopyrum*, *Prunus Pissardi*, etc. Kozłowski (261) also assumes that the anthocyanins in *Pelargonium zonale* result from oxidation of flavonols.

From smaller structural elements. Numerous authors assume a biogenesis of anthocyanins from smaller structural elements than the flavones and allied substances. Robinson (446, 449, 452) assumes that anthocyanins, flavones, flavonols and related substances, all of which show in the aglycon 15 C atoms, are formed from a $C_6-C_3-C_6$ structural framework. Both of the C_6 rings are aromatic in nature. Ring A usually occurs as phloroglucinol, Ring C in the form of catechol. Both rings are built of hexoses and are bound together by a triose by means of aldol condensations with formation of a hypothetical intermediate product (XLV):



Condensation of the ring C with the triose occurs first. Afterwards the adjunction with Ring A takes place. In this connection the author points out that substances with a C_6-C_3 structural frame-

work are widely distributed in nature, for example, eugenol, coniferyl alcohol, *etc.*

The hypothetical intermediate product (XLV) can easily be transformed into various end-products by means of oxidation, subsequent dehydration and ring closure. Oxidation at C_1 leads to formation of cyanidin (XLVI), at C_3 to flavone luteolin (XLVII) and at both C_1 and C_3 or at C_2 and C_3 to flavonol quercetin (XLVIII). Owing to this mechanism of oxidation, which appears probable also from other investigations (229, 276), the possibility exists of simultaneous formation of anthocyanins and anthoxanthins in the plant. In point of fact, the corresponding flavonols and anthocyanins are often present together in the same plant, for example, cyanin with quercetin (109, 110).

Genetic research (*e.g.*, 298) has shown that anthocyanins and anthoxanthins are actually produced simultaneously. The formation occurs from two plant substances. One of these substances is present only to a limited extent and contained in all formed pigments as a structural element. The second component is produced in amount and variety dependent on the factorial influences and interactions. According to the ideas of Lawrence and Scott-Moncrieff, the first substance, available only in limited quantity, would represent the nucleus A, the second the C_6-C_3 structural framework in one of its stages of development. These results agree to a high degree with the hypothesis of Robinson, and thus represent important proof for Robinson's idea, expressed long ago, from a totally unexpected field of investigation.

It may be seen from the numerous investigations on the systematic distribution of the anthocyanins (297) and of the anthoxanthins (130), that, in anthocyanins as in anthoxanthins, the natural pigments, which possess a hydroxyl group for each of the C atoms 3' and 4' in the ring C, are the most frequent. The catechol nucleus as a C ring is therefore the most wide-spread; cyanidin and quercetin are, if this hypothesis is correct, the simplest members of the anthocyanidins and flavonols which the plant can form. For formation of delphinidin and myricetin or pelargonidin and kaempferol, further oxidation or reduction would therefore be necessary.

Rao and Seshadri (412) have recently accepted Robinson's hypothesis for the biogenesis of the anthoxanthins.

Hibbert (184) has expressed ideas similar to those of Robinson

concerning formation of these pigments. However, he is of the opinion that the C_6-C_3 unit is formed from a condensation reaction between phenols and methyl glyoxal.

Freudenberg (109, 110) looks upon the anthocyanins, flavones, *etc.*, as derivatives of α,γ -diphenylpropane, formed from cinnamic aldehydes and oxyphenols. Provided one does not take into consideration the hydroxyl groups of the two benzene rings, the difference between the anthocyanins, flavones and allied substances are based on the differing stages of oxidation of the three C atoms lying between the benzene rings. The fact that anthocyanins, flavones and flavonols with the same arrangement of the hydroxyl groups in the rings A and C are found in the same plant, is regarded by Freudenberg as a support for this hypothesis.

Reichel and Schickle (416) infer, on the basis of results obtained from model experiments, that anthocyanin-similar compounds are formed in nature by condensation of the glycosides of the oxyacetophenones with the glycosides of the oxy-benzaldehydes. The wide distribution of such preliminary stages in the vegetable kingdom, necessary for the condensation, is considered by these authors as an evidence for their hypothesis.

From the de-amination products of decarboxylated amino-acids. Mrs. Onslow (370, 371) is of the opinion that formation of anthocyanins, flavones, *etc.*, is narrowly connected with protein metabolism. On the basis of physiological considerations she assumes that anthocyanin pigments are produced from the residues of aromatic amino-acids after de-amination. Frey-Wyssling (112) looks upon the de-amination products of decarboxylated amino-acids, in general, as the source of many secondary plant products (alkaloids, terpenes, *etc.*). Formation of anthocyanins is regarded by this author as being due to these metabolic processes.

From leuco-anthocyanins. Leuco-anthocyanins are also regarded as preliminary stages in the formation of anthocyanins. Jonesco (209) regards the leuco-anthocyanidins as precursors of the anthocyanidins. Bancroft and Rutzler (18) draw the inference that the red of the copper beech comes probably from a leuco-anthocyanin. Rutzler (460) likewise regards the leuco-anthocyanins together with the flavones as probable precursors of the autumn colour.

Scott-Moncrieff (492) points out, however, that the relations

between leuco-anthocyanins and anthocyanins are not as simple as they would seem to be at first glance. She believes that—apart from a few exceptions—the leuco-anthocyanins are to be regarded rather as the end-products of parallel syntheses from a common pigment precursor. The inter-conversion of any of these pigments *in vitro* is in no way evidence of similar biosynthetic processes.

* * *

To date, experimental proofs for the correctness of the hypotheses mentioned above, have not appeared in any great quantity. Nevertheless, it seems to be fairly well excluded that anthocyanins are formed in the plant by reduction of flavonols. On the other hand, it is certain that oxidation processes play an important rôle in the formation of these pigments, as is suggested in the hypothesis of Robinson. This hypothesis is based, however, largely on the results of genetical investigations. It can also be looked upon as a basis for a natural explanation of the systematic distribution and genetic transmission of the various anthocyanins.

The question as to what rôle is played by the leuco-anthocyanins in the metabolism of the anthocyanins, is to date too unsettled to permit final judgment at present.

*Relation between Formation of Anthocyanins, Sugar and
Nitrogen Metabolism*

On the basis of his experiments on anthocyanin formation using artificial nourishment by means of various kinds of sugars, Overton (372) came to the conclusion that the appearance of red cell sap was in close relation to the sugar content of the cell sap. This assumption has found many adherents even to-day, though it has never been proved with exact methods.

Others (75, 203) have tried to prove Overton's hypothesis quantitatively. The results, positive in this regard, which they obtained, can not be looked upon, however, as support for Overton's theory, owing to reasons of method. The same applies to histochemical findings (229) concerning an intimate connection between starch, sugar and anthocyanin in the seedling of *Fagopyrum esculentum*.

Some investigators (255, 256, 345, 350, 425) assume a close relation between anthocyanin formation and the quantity of as-

similates, *i.e.*, sugar. Gleisberg (131), on the other hand, obtained no clear results in his experiments with cane sugar as a nutritive solution. Griffin (136), too, is unable to find that there is a pronounced dependence of pigment formation on sugar content.

Exact researches on the connection between sugar metabolism and anthocyanin formation in seedlings of red cabbage have shown no such intimate relation (116) as that assumed by Overton and many other authors. It is true that in the seedlings, which in general contain more sugar, more anthocyanin was formed as well. But a comparison of individual results is unfavourable for the "sugar theory". The lack of any regularity whatsoever in the relation between anthocyanin and sugar content in the individually investigated organs as well as in the whole seedling, renders a quantitative relation between sugar and anthocyanin content highly improbable.

Lippmaa (308, 309, 310) succeeded, by means of artificial feeding with sugar, in increasing the formation in different plants not only of anthocyanins but of carotenoids as well. In his experiments chloroplasts changed into chromoplasts and thus gave the leaves of the plant in question a significantly darker appearance. According to Lippmaa, sugar is of importance as a precipitating factor in the formation of anthocyanin, as is assumed by others (123, 351). Lippmaa sharply rejects the idea of a connection between sugar metabolism and anthocyanin formation.

Noack (365) believes that formation of anthocyanins after sugar addition can be traced to a destructive effect on the chloroplasts or on the assimilation of accumulated sugar in the tissue. He is of the opinion that other factors which promote formation of anthocyanins (temperature, lack of mineral substances, *etc.*) can be explained in this way as well.

It may be pointed out in this connection that the content of the plant in other aromatic compounds can be increased by use of a nutritive solution rich in sugar. Lang (289) has made such observations with naphthochinones and tannins. Danner (86) was able to increase significantly the arbutin content of his experimental plants (*Saxifraga*) by artificial sugar feeding.

Less recent authors have often assumed a connection between anthocyanin formation and nitrogen metabolism. Literature in this connection can be found in the publications of Mrs. Onslow

(369). In our own experiments we were unable to find any support for this hypothesis, using seedlings of red cabbage (116). Gleisberg (131) even found a decrease in anthocyanin formation upon increased nitrogen feeding, whereas Schulz (485) found more anthocyanin in barley kernels having more proteins.

If it be true that there is a connection between sugar and nitrogen metabolism, on the one hand, and anthocyanin formation, on the other, then it is probably of only secondary importance (for example, artificial sugar nutrition—or nitrogen nutrition → increased metabolism → increased anthocyanin formation).

So far, quantitative investigations are almost entirely lacking on these very questions. Such research, however, would do a great deal to clear up the question of the relation between sugar and anthocyanin metabolism.

Behaviour of Anthocyanins During Hunger Metabolism

Kuilman (276) made the observation that the anthocyanin content of *Fagopyrum esculentum* seedlings decreased when they were kept in the dark. Frey-Wyssling and Blank (115, 116) noticed similar decrease with seedlings of *Brassica oleracea* var. *capita rubra*, which showed a constant decrease in anthocyanin content at 10° just as at 20° and at 30° C. The sugar content of the seedlings decreased with considerably greater rapidity, however.

To what extent anthocyanin is reformed when metabolism begins again, is a question that can not yet be answered. It is perfectly possible that with seedlings of red cabbage, degradation of the anthocyanins goes only as far as the colourless preliminary stage (a glycoside). Seedlings deprived of nutrition start to show pigment formation after the culture has been sprinkled with hydrogen peroxide, first in the lower part of the hypocotyl (116).

The hungering plant certainly does not consume the pigment in its metabolism because of the glycoside-bound anthocyanin sugar. The latter is present in the seedling in only extremely small quantities, since, as is well known, only a third of the anthocyanin molecule is composed of sugar. The ratio of total mobilisable reserves of carbohydrates to glycosidic-bound sugar content is so vast that even in hunger metabolism the sugar of the anthocyanin molecule can hardly be of significance as a source of energy (reserve substance) (116).

Jonesco (204) has already pointed out that anthocyanins may not be regarded as a "substance de déchet". The plant is able to use them at any moment in its metabolism. We should like to concur in this opinion. In this regard the anthocyanins behave like other secondary plant substances. Thus *Parthenium argentatum* takes up a part of the caoutchouc formed by it into its metabolism anew (511). Mazanko (330) has made the same observation with *Taraxacum megalorrhizon* under different experimental conditions. The alkaloids of *Lupinus luteus* (545) and the essential oils of *Salvia officinalis* (114) behave in the same way.

Concerning the question as to what rôle the anthocyanins and other secondary plant substances play in the starving plant, we are forced to remain unenlightened so long as we are not successful in obtaining the conversion products resulting from them.

Factors Affecting Formation of Anthocyanins in Plants

Influence of light. Favorable influence of strong illumination in promoting formation of anthocyanins has been observed in *Chrysanthemum* and *Abutilon* (254, 257), in Geraniaceae (591) and *Coleus* (103) and in *Diervilla* (236).

Chi-Yuen-Chia (65) was able to attain a significant decrease of anthocyanin content in *Amaranthus odoratus* by decreasing illumination. Continuous illumination caused a discontinuation of formation of the pigment in his experiments. This last fact is in good accord with results obtained by others (229, 276). Both of these authors agree in emphasizing the fact that a photochemical as well as a darkness reaction are necessary for the formation of anthocyanin in *Fagopyrum esculentum*.

Pearce and Streeter (375) showed that the region from 3,600 Å to 4,500 Å of the solar spectrum is most influential in colouring apples. They suggest that a flavonol is transformed by light energy into the red pigment. Arthur (10, 11) investigated different artificial sources of light in regard to their effects on formation of anthocyanin in apples. The best light source for colouring apples was found to be mercury vapour arc in Uviol glass. Formation of the pigment takes place only in the living cells of the epidermis of the apples.

Allen (3) was able to accelerate the formation of anthocyanin in plums by means of illumination. However, anthocyanin is also

formed when sunlight is excluded. On the other hand, apples, apricots, pears and peaches all require sunlight for the formation of their anthocyanin. For this reason peaches do not take on a red colour in storage. Similar observations have been reported elsewhere (4).

According to Semmens (494) polarized light is able to change anthocyanin into anthocyanidin in germinating seeds of *Tropaeolum*, *Fuchsia*, *Geranium* and other genera. In the experiments of Braun (40) ultraviolet rays injured the plants and brought about formation of anthocyanin. Others have described X-ray-induced modifications of flower colour in *Petunia*, *Nicotiana*, *Phlox* and *Salpiglossis* (200, 352), and Biebl (29) is of the opinion that his experiments show that plants containing anthocyanins have a greater resistance against ultraviolet rays. Bünning (47) also attributes to the anthocyanins a protective function against ultraviolet rays.

Formation of anthocyanins in the dark. According to Kuilman (276) and Karstens (229), a photochemical reaction is also necessary for production of anthocyanin in the plant. Nevertheless, there are plants in which formation of this pigment takes place even during exclusion of all light. This is true of Crassulaceae, Saxifragaceae, Compositae, Balsaminaceae and Melastomaceae, in which anthocyanin is produced in the vegetation point of the root (346). Bünning (48) also was able to observe formation of anthocyanin in the dark. Seedlings of red cabbage take on colour by means of anthocyanin formation likewise without any illumination whatsoever; they become reddish-violet (116).

A similar phenomenon, namely, that it depends on the plant whether light is necessary for the formation of pigment, can be found in the synthesis of chlorophyll. Here, too, there are plants (seedlings of Coniferae; algae when there is artificial protein feeding; etc.) which do not require light for the formation of chlorophyll. The great majority of plants, however, are unable to form their green colouring matter without illumination.

Influence of temperature on formation of anthocyanins. Many investigations have been undertaken concerning the influence of temperature on anthocyanin formation (105, 116, 123, 257, 260, 276, 351, 549, 591). Since in the majority of cases the pigment was not extracted and then quantitatively determined, the great many contradictory reports are understandable. Estimation of the pig-

ment content in a plant is liable to great sources of error, since the colour is also influenced by the reaction of the cell sap, the carotenoids, co-pigments, etc.

Several investigators (123, 257, 276, 351, 469, 591) draw from their studies the conclusion that low temperatures have a favourable influence on the formation of anthocyanins in general. Weisse (549), working with *Pelargonium* and *Geranium* species, observed the opposite effect. The investigations of Harder and co-workers, which will be dealt with in another connection, also often showed an increase in anthocyanin formation at higher temperatures.

Frey-Wyssling and Blank (116) have followed the formation of anthocyanin quantitatively in seedlings of red cabbage in the dark at temperatures of 10°, 20° and 30° C. At 20° and 30° C. the anthocyanin content was much higher than at 10° C. At 30° C., however, a noticeable decrease in the pigment content of the germinating seed started to set in. The optimum temperature for anthocyanin formation lay in this case probably between 10° and 30° C.

Apparently all plants have an optimal temperature for anthocyanin formation. This probably coincides with the optimal temperature for metabolism.

More accurate insight concerning the effect of temperature on pigment formation will be attained only when further quantitative investigations on pigment formation at various temperatures have been carried out and reported.

Anthocyanin formation as a result of foodstuff deficiency. Plants which have a foodstuff deficiency often show increased anthocyanin formation. Steinecke (516) found a large quantity of anthocyanin in *Lathyrus* and *Viola* species growing on sand dunes particularly poor in foodstuffs. Sugar beets, as revealed in extensive research material (264), often show increased formation of red or violet pigments during deficiency conditions. Lettuce shows the same tendency (541, 586). Tomato is very sensitive to phosphorus deficiency (333); when this nutritive element is deficient, the lower side of cotyledons and foliage leaves show an especially high content in anthocyanin. Calcium deficiency can also be the cause of an increase in pigment formation (317). Berthold (28) states, together with Boysen Jensen (36), that maize reacts to foodstuff deprival by a stronger formation of anthocyanins. Gassner and

Straib (124) have investigated the formation of anthocyanin in young barley plants with deficiency of phosphorus, potassium and nitrogen. They are of the opinion that increase in pigment formation may be explained quite naturally as a result of the amount of available carbohydrates. Red coloration is also promoted by addition of potassium to the diet of red cabbage, whereas nitrogen and phosphorus additions decrease the pigment content (418). That potassium deficiency accelerates anthocyanin formation is apparent beyond all doubt from one compilation (93) and from an investigation (544).

Anthocyanin formation in infected and injured plants. Many authors describe an increase in anthocyanin formation in plants attacked by parasites, in infected plants and those which have suffered some sort of injury. Steinecke (516) noticed formation of anthocyanin in leaves, caused by aphids. Küster (267) found anthocyanin in the supporting tissue of galls and in infected plants. Gertz (128) also noticed its presence in *Quercus* galls. Bodmer (33) observed how species of thrips stimulated anthocyanin formation in the pollen of *Lythrum Salicaria*. Similar observations have been made when a species of *Beta* was attacked by *Cercospora beticola* (78), on corn plants attacked by *Ustilago zeae* (70, 156) and on *Melandrium album* infected with *Ustilago violacea* (32). Lippmaa (312) also reports an increase in anthocyanin formation after mould infection. Longley describes (314) the distribution of anthocyanin in tulips after they had been infected with mosaic disease.

Injured corn plants have manifested increased formation of pigment (315), and indole-3-acetic acid injected into *Ricinus* plants promoted formation of anthocyanin not only at the point of injection but also in the tissues of the medulla (510). Increased formation of anthocyanin was also observed when apples were sprinkled with thiocyanates (92).

Although, in general, formation of red and violet pigments in infected and injured plants may be attributed to anthocyanins, there are two investigations in which the pigments formed upon infection did not turn out to be identical with anthocyanins. Nierenstein (363) found in a chemical examination of the pigment from the red pea gall on *Quercus pedunculata* that it had no relation to the anthocyanins. Petrie (377) also could find no trace of anthocyanins

in the leaves of *Eucalyptus stricta* which had been attacked by *Eriophyes* and subsequently showed strong red coloration. The red and violet pigments in diseased plants are obviously not identical with anthocyanins in every case. It is possible that a more exact investigation of such plants would show that in other cases as well anthocyanins are not the cause of red coloration.

Formation of these pigments after injury and infection is probably to be attributed to a disturbance in the normal metabolism—similar to that in deficiency in mineral substances. The reports (419) that in plants infected with virus there are disturbances in the phenol and more especially of the tannin metabolism, are of particular interest in this connection.

It is quite within the realm of possibility that pigments can be produced from leuco-anthocyanins in diseased plants (452).

Photosynthesis in Leaves Containing Anthocyanins

Chlorophyll content of red leaves containing anthocyanins. Willstätter and Stoll (579) long ago observed during the course of their fundamental investigations that the red foliage leaves of *Acer pseudoplatanus* contained less chlorophyll per unit area than leaves of the green variety. Kuilman (276) has made the same observation on the leaves of *Corylus maxima* f. *atrosanguinea*, *Malus pumila*, *Chaenomeles japonica*, *Berberis Neuberti*, *Deutzia crenata*, etc. On the other hand, the red leaves of *Paeonia albiflora* contain more chlorophyll than the green ones. But red leaves of *Eranthemum* (495) and *Corylus avellana* (498) contain less chlorophyll than green ones.

Absorption of light through leaves containing anthocyanins. Capeletti (55) makes anthocyanin responsible for the greater absorption of ultraviolet rays in red leaves, and several such leaves of *Fagus sylvatica* layed one over the other are able to absorb almost the whole of the visible range of radiation (466). Kosaka (259) is of the opinion that the increased activity of transpiration in plants containing pigments is due to increase in absorption. A 10% higher light absorption in the middle range of the spectrum has been noted in anthocyanin-containing sun leaves of *Corylus* (498), and considerably lowered permeability for light rays has been detected in ecological surveys of South Africa (137).

Temperature of leaves containing anthocyanins. Red leaves of

Eranthemum have a higher inner temperature than green ones (495). Alsaç (6) found only narrow differences in temperature in his investigations with *Rhoeo discolor*, *Vriesea splendens* and *Ficus variegata*. Using a 200-watt lamp for purposes of radiation at 50 cm. distance, the difference amounted to only 0.5° C. between the red lower side and the green upper side. This author assumes that anthocyanins are of no importance to the plant from the point of view of thermal economy. So far, no positive selective effect has been noticed in plants containing anthocyanins.

Photosynthesis in leaves containing anthocyanins. Leaves of purpurea varieties assimilate almost as intensely as do green leaves, if one brings the intensity of photosynthesis into relation with chlorophyll content (276, 579). Very often the difference in intensity of assimilation between red and green leaves can be explained by a difference in the amount of chlorophyll contained by them. As in young green leaves, young red leaves also manifest an increase in the "Assimilationszahl".

In some cases Kuilman (276) was even able to observe an increased assimilation in red leaves. Sen (495) also noticed more intense photosynthesis in the red types of *Eranthemum*, although the chlorophyll content was greater in the green leaves.

Gabrielsen (121) observed during the course of his investigations a filter effect of anthocyanin in foliage leaves, tending to decrease assimilation. This phenomenon can be noted in all ranges of the spectrum. That author also investigated assimilation in leaves containing anthocyanin when irradiated by light of different wave lengths. He is of the opinion that the differences between his experimental results and those of Willstätter and Stoll (579) can be attributed to the difference in intensity of radiation to which they were subjected. In the experiments of Willstätter and Stoll, the radiation was too strong to permit notice of an absorption effect.

According to the same author, in nature the difference in assimilation in red and green leaves is greatest when they are exposed to light from a blue sky, and least when they are exposed to the light of early sunrise or sunset, *i.e.*, when the sun is low on the horizon.

Autumnal leaf coloration by anthocyanins. Lippmaa (311, 313) has made some remarkable observations with about 250 varieties of plants in Esthonia and the Altai with regard to the temporal ap-

pearance of anthocyanins and plastid pigments. The most important result of this investigation is that autumnal formation of anthocyanin appears exclusively in those varieties of plants whose young leaves already possess the capacity for anthocyanin synthesis. In autumnal anthocyanin formation this author sees an automatic repetition of the "redness of immaturity and dearth". The anthocyanin formation begins in leaves rich in chlorophyll, and in the beginning can hardly be noticed. Later it runs parallel to the chlorophyll degradation. The capacity for building anthocyanin is present until death of the foliage. Lippmaa is of the opinion that no ecological significance is to be attached to autumnal leaf coloration by means of anthocyanins.

Stanescu (514) believes that autumnal formation of anthocyanin takes place at the cost of the starch reserves.

Molisch (345) assumes, on the basis of his observations in Japan, that anthocyanin formation is dependent on accumulation of sugar in the leaf. On the other hand, he regards the anthocyanin formed in the autumnal leaf as a waste product of metabolic processes. He compares it with silicic acid, the calcium salts of oxalic and carbonic acid, as well as with tannins, which, contrary to proteins, potassium and phosphorus compounds, do not return to the plant.

Other work (276) indicated that yellowing of leaves of *Quercus rubra* occurred simultaneously with red coloration. The "Assimilationszahlen" of these leaves became greater, for no decrease in assimilation occurred concurrently with degradation of the chlorophyll. Leaves of *Liquidambar styraciflua* behaved in the opposite sense. Nevertheless, in both cases the intensity of the assimilation was surpassed by that of the green leaves. This difference was reduced to a minimum, however, in *Deutzia crenata*. Kuilman, however, by no means draws the conclusion from these results that the presence of these anthocyanins is connected with lower assimilation coefficients.

Significance of Anthocyanins in Plant Metabolism

So far, the most varied opinions have been expressed concerning the physiological significance of anthocyanins in the vegetative organs of plants.

Owing to their optical properties, they are claimed to have a

favourable influence on enzymological processes and on chlorophyll formation (308). Seybold (497), on the other hand, regards the anthocyanins as optico-physiologically unimportant; according to this author, their formation is nothing but the expression of physiological disturbances.

Noack (365) attributes a rôle in the reduction process of carbon dioxide assimilation to the flavonol-anthocyanin system. He compares this function to that of the respiratory chromogene pigment in sugar fermentation, as shown in the investigations of H. Wieland. Kosaka (258) claims to have noticed an increase in assimilation in plants containing anthocyanin. Kuilman (276) was unable, on the basis of his experiments, to observe either a physical or a chemical relation between anthocyanins and carbon dioxide assimilation. Molisch (345), too, rejects the idea of participation of anthocyanins in carbon dioxide assimilation, especially in view of the autumnal coloration of foliage leaves by anthocyanins.

Stiles (517) assumes that anthocyanins can play a rôle in the respiration of plants as glycosides. Zanoni (592, 593, 594) observed increased respiration and an increase in basal metabolism in the leaves of red varieties. This fact is attributed to increased photosensitivity of the red tissue owing to anthocyanins.

Others (49, 414, 415) look upon the anthocyanins as hydrogen acceptors in fermentation processes. Once their task has been completed in the redox system, they then, especially in autumnal foliage leaves, are transformed to catechines.

Even though one-year-old plants have been claimed to make use of their glycosides often as reserve substances, and those of several years to do so only rarely (39, 41), the investigations so far carried out and mentioned before (116) by no means point to such a function.

Frey-Wyssling (113) regards the anthocyanidins as eliminated metabolic products which are glycosided and thus made water-soluble allowing their excretion into the cell sap. Jonesco (204), on the other hand, is of the opinion that the anthocyanins can by no means be regarded as waste products.

All these hypotheses are hardly able to give a satisfactory explanation of the physiological significance of anthocyanins in vegetative plant organs. The desire alone to attribute a function to these pigments, which are formed in the most varied plant organs

under the most differing conditions, involves difficulties of no mean order. Nevertheless, it may be hoped that new investigations, especially quantitative ones, will enable us to obtain deeper insight into the physiology of anthocyanins, and thus approach nearer to the riddle of the "vegetable chameleon".

Natural and Artificial Alterations in Flower Colour and the Flower Colour Pattern

Natural alterations in the colour of flower petals. The especially interesting natural alterations in the colour of flower petals, which depend mostly on the formation and characteristics of the anthocyanins and their accompanying substances, make their appearance in the temperate as well as in the tropic zones. In the latter they attract more attention, as there the changes in colour take place much more rapidly and for this reason are much more striking.

Winkler (582) investigated the course of flowering in *Helicteres isora* L. in Cameroon. The grey-bluish petals first became violet at anthesis and then bright red. The sap markings were at first black and then became brown. Howard and Howard (188) observed colour alteration in the petals of *Hibiscus Sabdariffa* L.

Cammerloher (54) was able to observe colour change during anthesis in the petals of *Bauhinia scarlatina* in Java. When the flowers opened, the petals were orange and afterwards became fire-red. Molisch (348, 349) has described numerous examples of colour alterations appearing naturally in flower petals in British India, for example, in *Hibiscus mutabilis*, *H. tiliaceus*, *Capparis horrida*, *Quisqualis indica*, *Franciscea latifolia* and *Datura Metel*. The colour changes are from white to red or from blue-violet to snow-white. They depend on the presence of free oxygen. White flowers show no red coloration when submerged in water. Only upon addition of hydrogen peroxide is the red coloration brought about by formation of anthocyanin.

In Sumatra, Kuijper (275) made a more exact study of the well known colour alteration in petals of *Hibiscus mutabilis*. The white flowers open only at 4 o'clock in the morning and remain white until 9 o'clock. They then gradually become reddish, and at 17 o'clock are quite red. From then on they slowly close, and the next morning they are dark red and wilted. Kuijper was able to obtain a flavone-like compound from these white flowers, which

could be reduced to an anthocyanin by means of powdered zinc and magnesium. He infers that pigment formation in the flower takes place in a similar fashion. Lowering of the temperature inhibits the colour change both in light and in darkness, whereas at normal temperature darkness accelerates the colour change in the petals of the buds.

To what extent, however, different plants behave differently in this connection, is shown by other reports (520, 521). Smith (378) has measured the pH of several plants which change their colour during the course of development. *Ipomoea Leerii* has, for instance, a diurnal colour-range from magenta-pink (bud) to full blue in the freshly-opened flower, corresponding to a pH range of 6.0-7.8. Development of the full blue is conditioned by both light and temperature. Robinson (438) has investigated the natural colour change more exactly in a number of flower petals. Unfortunately, only a few of her results can be stated here; they permit a certain insight, however, as to how the plant can effect such colour alterations. The cell sap of young red buds of *Anchusa* has a pH of 6.35; the blue petals which develop from these, however, have a pH of only 6.2. Both in the young red buds and in the blue petals the content in flavone was the same in quantity; the anthocyanin content, on the other hand, was greater in the red buds. Blue petals or parts of petals of *Delphinium* (pH about 5.6) contain less anthocyanin and less flavone than violet petals (pH about 5.7) of the same plant. Here colloid association is indicated. The concentration of anthocyanins in purple flowers of annual cornflower is higher than in the blue flowers. Red and blue flowers of *Hydrangea* contain the same anthocyanin, namely delphinidin 3-mono-side. There is a little more flavone in the red than in the blue flowers. The ratio of anthocyanin concentrations, red to blue, is usually from 6:1 to 7:1. The pH of *Hydrangea* is variable, the red flowers being slightly more acid than the blue.

An observation of Tammes (526) may also be mentioned at this point. This authoress was able to detect a colour alteration in the petals from white to red only in heterozygotic forms of *Dianthus barbatus*, a fact which is of especial interest.

Colour change in petals is still not clear from the physiological and cytological points of view, but presents a field in which there are many opportunities for interesting investigations.

Artificial alteration of flower colour pattern. The alterations of flower colour pattern mentioned above take place during the natural development of the flowers. Baur (19) observed some time ago that high temperature inhibits formation of anthocyanins in *Primula sinensis rubra*, which then develop white flowers.

Harder and his co-workers (106, 157, 158, 159, 160, 161, 162, 323, 483, 518, 585) have carried out numerous investigations on the question as to how the petals of different flowers blooming successively on the same plant are influenced by external factors. They (159) found that *Dahlia variabilis* produces yellow flowers when growing in the open, but red ones in the hothouse at 30° C. The buds which bloomed to red flowers were 0.5–1.5 cm. long during the temperature treatment. The period of sensitivity, during which formation of the red anthocyanins can be influenced, begins about a month before blooming and ends about ten days before full opening of the flower. In a continuation of these researches, plants were investigated, as well, whose petals do not show a homogeneous distribution of anthocyanin, but are patterned. In *Calliopsis bicolor* (323) high temperature at first causes progressive translation of the anthocyanins towards the points of the coronal leaves; finally the petals become so rich in anthocyanin that the flowers seem to be monotone brown. *Calceolaria hybrida grandiflora* (106) behaves quite differently. High temperature suppresses formation of the anthocyanin, so that finally pure yellow flowers are produced. In *Hibiscus mutabilis*, *Diervilla coraeensis*, *Victoria regia* and *Lantana hybrida* pigment formation is also controlled by temperature. Light intensity (483), too, plays an important rôle in the colour pattern of flowers in *Petunia*.

Whereas sensitivity is present during a very early stage of development and can be influenced by external factors, anthocyanin formation begins in *Petunia* only in buds of 5–10 mm. length. With similar belatedness are formed the precursors from which the anthocyanin is to be built up (518). In the non-white areas of the *Petunia* flowers the presence of a flavonol could be proven in young buds which still had no anthocyanin. However, the former was not present during the period of sensitivity.

The transformation of chromogen to anthocyanin is linked to a definite gene in *Petunia* (518, 585). If this gene is missing, the flavone-like compounds are not transformed to anthocyanin.

Unfortunately, the attempts of Harder to isolate the substance which regulates all these pattern formations have not as yet led to any tangible result.

*Significance of Anthocyanins with Respect to the
Flower and Its Biology*

The biological significance of colour to the flower, first recognized by Sprengel (513), was long disputed. For a period of time it was even denied, owing to the supposed total colour-blindness of bees. Proof that bees and other flower-seeking insects are able to see and recognize colours has since been perfectly established by zoologists (117, 195, 266, 271, 316, 472, 482). Later, botanists as well became interested in the biological significance of pigments, especially the anthocyanins, to the flower.

Knoll (241, 242) was able to observe a striking difference in the formation of pigments in *Muscari racemosum*. The fertile flowers of these plants possessed anthocyanin in more cell layers than the sterile flowers. The same author (243) was able to demonstrate by means of his detailed studies that the great intensity of blue-violet and yellow colours attracts *Deilephila livornica*. A greater sensitivity to colour than to odour has been demonstrated in the investigations on *Protocarpae convolvuli* (244). Bumblebees also have a pronounced sense of colour (270, 271, 272, 273).

In contradistinction to the bees, birds are able, as is well known, to perceive red. Porsch (385) has observed that petals coloured bright red by anthocyanin appear especially in those plants which are fertilized by birds.

It can be seen from certain extensive researches (297) that cyanidin is strikingly distributed in various leaves, fruits and flowers. Amongst 100 genera investigated, the following numbers contain it:

Autumn leaves	95
Young leaves	93
Permanently pigmented leaves	80
Fruits	69
Flowers	50

These figures indicate that the colour of flowers and fruits is of some importance for the attraction of insects and birds and that by means of natural selection a greater variation of the anthocyanins has developed in these organs.

In connection with certain data (295, 385) it would be of great interest to establish, by means of exact statistics, which insects or birds frequent the flowers investigated. Analysis of the various coloured parts of petals, as carried out long ago on *Papaver Rhoeas* (475, 476), would complete these investigations. We are of the opinion that the results obtained from such researches not only would be further verification of sensitivity to colour, but would also bear out the observations made by Sprengel 150 years ago. They might also perhaps prove the ideas maintained (295) on the greater variation of anthocyanins in flowers and fruits, as well as render differences in geographical distribution of the flower pigments understandable (23).

GENETICS OF ANTHOCYANINS

The study of heredity with respect to anthocyanins shows important results and has achieved successes which can claim general interest far beyond the special field in the narrower sense of the word. The beginning of a series of important investigations can be traced back to Onslow (369, 371) who began her experiments even before the chemistry of anthocyanins had been made clear. But it was only after progress had been made in the chemistry of these pigments through the researches of Willstätter, Karrer and Robinson, especially the development of qualitative tests by R. Robinson, that these genetic investigations could be successfully undertaken. The stimulus which J. B. S. Haldane gave to Mrs. Scott-Moncrieff (154) fell on fertile soil. Since then, a whole series of investigations has arisen from the worthy collaboration between English chemists (Dyson Perrins Laboratory, Oxford) and geneticists (John Innes Horticultural Institution, Merton Park) (20, 21, 22, 23, 24, 80, 122, 291, 292, 293, 298, 304, 307, 341, 380, 381, 470, 488, 489, 490, 522, 583).

The special value of these researches resides in the fact that not only the colour as a whole was followed in the process of heredity, but that also the inheritance of chemical individual substances was studied. This led to the winning of important evidence concerning the biosynthesis of the anthocyanins.

A few writers (295, 490-492) have compiled the chief results of these investigations, and we shall deal with them only very briefly. Investigations on the heredity of anthocyanins in flower petals are concerned with

- a) genes controlling pigment production
- b) genes controlling modification in anthocyanin type
- c) genes controlling the condition of the cell sap

To date, the following results have been obtained (490):

1. Plastid pigment, co-pigment, anthoxanthin and both general and specific anthocyanin production are generally dominant to their absence, but rarely is a dominant inhibiting factor involved.

2. Modification involving more oxidised anthocyanin pigmentation is dominant to less oxidised, and more methylated pigmentation to less methylated.

3. 3-5-diglycosidic and acylated (complex) anthocyanin pigmentation are dominant to 3-monoglycosidic and normal anthocyanin respectively.

4. More acid petal pH is dominant to less acid.

5. Uniform pigment distribution is generally dominant to flaking or marbling, or to picotee harlequin or delilah types, but it is common to find the wild type carrying a gene for slight anthocyanin inhibition over part of the flower.

The question as to how acylation of the aglycones is controlled by genes has so far not been sufficiently investigated.

In many cases it could be demonstrated that only one gene controls pigment formation, cell sap reaction, etc.:

	VARIETIES		
	DOMINANT	RECESSIVE	FACTOR
I. Yellow plastid production (<i>Cheiranthus Cheiri</i>)	yellow brown	lemon purple	Y
II. Yellow anthoxanthin production (<i>Primula acaulis</i>)	yellow	ivory or white	Y
III. Ivory anthoxanthin co-pigment production (<i>Dahlia variabilis</i>)	bluish magenta	rosy magenta	J
IV. General anthocyanin production (<i>Antirrhinum majus</i>)	magenta or red	ivory	L
V. Specific anthocyanin production (<i>Papaver Rhoeas</i>)	crimson- scarlet scarlet	crimson- magenta pink or salmon	T
VI. Oxidation of anthocyanin aglycone (<i>Antirrhinum majus</i>) (3-rhamnobiosides) (<i>Papaver Rhoeas</i>) (3-biosides) <i>Cheiranthus Cheiri</i> (3,5-dimonosides)	<i>Cyanidin</i> magenta pink or crimson purple	<i>Pelargonidin</i> red salmon pink	B E P

VII. Oxidation and methylation of anthocyanin	<i>(Primula sinensis)</i>	<i>Malvidin</i>	<i>Pelargonidin</i>	
	(3-monosides)	red	coral	K
	<i>(Pelargonium zonale)</i>	pink	salmon	X
	(3-5-dimonosides)			
	<i>(Pharbitis Nil)</i>	<i>Peonidin</i>	<i>Pelargonidin</i>	
		purple	red	Mg
VIII. Local change in pH	<i>(Primula sinensis)</i>	magenta	blue	R
	<i>(Primula acaulis)</i>	red	slaty	S
	<i>(Papaver Rhoeas)</i>	scarlet	claret	P
		pink	mauve	

Other investigators (50, 151-153, 584) attained similar results in their genetical investigations on *Primula acaulis*, *Pharbitis Nil* and *Aster*.

Investigations on *Verbena* (22) have shown, compared with the data of the table given above, contradictory results. Pelargonidin derivatives were sometimes dominant, sometimes recessive to delphinidin derivatives. Monosides were sometimes dominant, sometimes recessive to dimonosides. Mixtures of anthocyanins occurred, due to incomplete dominance or to modifying factors.

The accounts of Beale (21) concerning dominance, together with those of Fedotov (100), are worthy of special attention.

A series of further investigations carried out in other institutes are likewise concerned with the heredity of anthocyanins:

<i>Antirrhinum</i> (467)	<i>Linum</i> (523, 525, 527)
<i>Aquilegia</i> (470)	<i>Lychnis</i> (16)
<i>Aster</i> (104, 584)	<i>Matthiola</i> (468)
<i>Beta</i> (90, 237)	<i>Mirabilis</i> (499)
<i>Brassica</i> (87, 524)	<i>Nicotiana</i> (507)
<i>Canna</i> (187)	<i>Oryza</i> (182, 193, 201, 202, 211, 358, 359, 360, 374, 407)
<i>Capsicum</i> (89)	<i>Papaver</i> (337, 362, 380, 381)
<i>Centaurea</i> (332)	<i>Pelargonium</i> (488)
<i>Cheiranthus</i> (122)	<i>Petunia</i> (303, 320, 321, 585)
<i>Coleus</i> (35, 424)	<i>Pharbitis</i> (151, 152, 153, 196, 197)
<i>Cosmos</i> (338)	<i>Phaseolus</i> (253, 287, 288, 339, 386, 387, 505)
<i>Crepis</i> (74)	<i>Pirus</i> (80, 307, 534)
<i>Dahlia</i> (291, 292, 293, 298, 528)	<i>Pisum</i> (100, 149, 550)
<i>Delphinium</i> (88)	<i>Plantago</i> (155)
<i>Dianthus</i> (526)	<i>Portulaca</i> (194)
<i>Godetia</i> (185)	<i>Primula</i> (50, 489, 583)
<i>Gossypium</i> (164, 165, 166, 167, 168, 189, 190, 191, 192, 239, 501, 502, 506)	<i>Ricinus</i> (163)
<i>Hibiscus</i> (188)	<i>Salpiglossis</i> (84)
<i>Hyoscyamus</i> (133)	<i>Secale</i> (533)
<i>Lactuca</i> (91, 531)	<i>Silene</i> (324, 325, 326)
<i>Lathyrus</i> (24, 405, 406)	<i>Sisyrinchium</i> (336)

<i>Solanum</i> (30, 31, 94, 118, 318, 353, 413, 462, 503)	<i>Tropaeolum</i> (341, 522)
<i>Sorghum</i> (408, 409)	<i>Verbascum</i> (262)
<i>Streptocarpus</i> (299)	<i>Verbena</i> (22, 24)
<i>Theobroma</i> (551)	<i>Vicia</i> (96, 504)
<i>Trifolium</i> (364, 552, 554)	<i>Viola</i> (72, 73, 108)
<i>Triticum</i> (134, 212, 233)	<i>Zea Mays</i> (42, 98, 199, 422, 423)

SUMMARY

Chemistry and biochemistry. The anthocyanin pigments of plants are glycosides, the sugar-free pigments or aglycons of which are called anthocyanidins. The anthocyanidins are derivatives of 2-phenylbenzopyrylium.

Proof of the constitutional formula of the anthocyanidins is founded on the conversion of flavonols into anthocyanidins, the fusion with potassium hydroxide, and various syntheses performed by R. Willstätter and R. Robinson and their co-workers. The aglycon of the red colouring matter of the flowers of *Pelargonium zonale*, pelargonidin, for example, is 3,5,7,4'-tetrahydroxy-2-phenylbenzopyrylium chloride.

By boiling the anthocyanins for a short time in 20% hydrochloric acid, the pigments may be split into anthocyanidin and the sugar components. The anthocyanins appearing in nature are partly mono-, partly di-glycosides. So far, glucose, rhamnose, galactose and gentiobiose have been isolated as sugar components. A number of plants contain anthocyanins in ester combination with an organic acid (malonic, p-hydroxybenzoic, p-hydroxycinnamic and 4-hydroxy-3,5-dimethoxycinnamic acid). Keracyanin from black cherries is, for instance, cyanidin-3-rhamnoglucoside and violanin from the flowers of *Viola tricolor* consists mainly of an acylated derivative of delphinidin.

The anthocyanins are extracted from well desiccated plant material by means of methyl alcohol containing 1-2 per cent hydrochloric acid. The anthocyanin chloride is precipitated with three-fold volume of ether. This purifying process is repeated according to necessity. As the anthocyanins usually appear as mixtures in various plants, the components of these mixtures may be separated by fractional crystallization of their picrates, or by use of the adsorption technique.

In solution, anthocyanins form red oxonium salts at pH 3; the violet form at pH 8.5 is represented by a colour-base of a quinoid

structure; the salts of this colour-base in a solution of pH 11 are blue.

An intimate chemical relationship exists between anthocyanins and other plant products. Quercetin, a flavonol, can be transformed to cyanidin chloride. The latter can be reduced to d,l-epicatechin; the reaction in the reverse direction may also be performed. Therefore, the anthocyanidins lie between the flavonols and catechins, from the point of view of degree of oxidation.

The various colours of tissues containing anthocyanins are not due alone to the various anthocyanins. The presence of anthocyanins in changing amounts and mixtures, alterations in the pH of the cell sap, the variable ash content of the latter, and above all co-pigmentation and the colloidal condition of certain components of the cell sap are all factors influencing the colour of the anthocyanins dissolved in the sap.

Nitrogenous anthocyanins have been isolated from plants of ten families. The structural formulae of these sensitive pigments have not yet been elucidated.

Leuco-anthocyanins have been found in plants. They are also glycosides, and in some cases may be regarded as precursors of the anthocyanin pigments.

Occurrence of the anthocyanins. So far, no anthocyanins have been detected in micro-organisms. They are present in the whole vegetable kingdom from the mosses upwards.

Using qualitative tests, developed by R. Robinson and his co-workers, an exhaustive survey has been made on the occurrence of anthocyanins and anthocyanidins in the plant realm. Apart from flowers, other anthocyanin-containing organs have been investigated.

The formation and presence of anthocyanins as a characteristic of classification is of importance for the division of many cultivated plants.

Pelargonidin derivatives predominate in the flowers of tropical and subtropical species. Delphinidin derivatives are the most common in temperate and alpine plants. Red-flowered forms have a greater survival value than blue ones in most tropical plants.

Morphology. In the majority of cases observed the anthocyanins are present in solution in the cell sap. They have also been ascertained in the cell plasma of *Allium Cepa* and in the cell walls of

mosses and of *Eichhornia crassipes*. A very peculiar phenomenon is presented by the formation of the so-called anthocyanophores, formation of which may be explained as the result of mixture separation.

Anthocyanins may be found in all organs of the plant from the root to the flower. They are often localized in the epidermis, the subepidermal layer and a few cells of the cortex.

Physiology. Formation of anthocyanins from flavonols and flavone-similar substances by reduction seems very improbable. According to R. Robinson, anthocyanins, flavones, flavonols and related substances, all of which show in the aglycon 15 C atoms, are formed from a $C_6-C_3-C_6$ structural framework. Both of the C_6 rings are aromatic in nature. Ring A usually occurs as phloroglucinol, ring C in the form of catechol. Both rings are built up of hexoses and are bound together by a triose by means of aldol condensations with the formation of an intermediate product. This intermediate product can be easily transformed into various end products (anthocyanins, flavones, flavonols) by means of oxidation, subsequent dehydration and ring closure. Genetic research presents important proof for Robinson's idea.

Exact researches on the connection between sugar metabolism and anthocyanin formation in seedlings of red cabbage have shown no such intimate relation as that assumed by Overton and many other authors. If it be true that there is a connection between sugar and nitrogen metabolism, on the one hand, and anthocyanin formation, on the other, then it is probably of only secondary importance (for example, artificial sugar or nitrogen nutrition \rightarrow increased metabolism \rightarrow increased anthocyanin formation). Further quantitative investigations would do a great deal to clear up the question of the relation between sugar metabolism and anthocyanin formation.

Seedlings of various plants show a constant decrease in anthocyanin content during starvation. As to what rôle the anthocyanins play in the hungry plant, we are forced to remain unenlightened, as long as we are not successful in obtaining the conversion products resulting from them.

A photochemical process is usually necessary for the production of anthocyanins in plants. Strong illumination often promotes the formation. Nevertheless, there are plants in which formation of these pigments takes place even during exclusion of all light.

Many investigations have been undertaken concerning the influence of temperature on anthocyanin formation. Since in the majority of cases the pigment was not extracted and then quantitatively determined, the great many contradictory results are understandable. Apparently all plants have an optimal temperature for anthocyanin formation. This probably coincides with the optimal temperature for metabolism.

Plants which have a foodstuff deficiency often show increased anthocyanin formation. Formation of anthocyanins in plants after injury and infection has not been proved.

Red foliage leaves often contain less chlorophyll. The same leaves also show an increased absorption of light. The differences in temperature between green and red leaves after illumination are only narrow. They seem to indicate that the anthocyanins are of no importance to the plant from the point of view of thermal economy.

The results of investigations in the rate of photosynthesis of coloured leaves are contradictory. They can be attributed to the difference in the intensity of radiation to which the leaves were subjected.

Autumnal formation of anthocyanins appears exclusively in those varieties of plants the young leaves of which already possess the capacity for anthocyanin synthesis. So far, quantitative investigations regarding autumnal formation of these pigments are almost entirely lacking. Their ecological significance is contested.

All the hypotheses expressed concerning the physiological significance of anthocyanins in the vegetative organs of plants (reserve substances, eliminated metabolic products, hydrogen acceptors, *etc.*) hardly give a satisfactory explanation. The desire alone to attribute a function to these pigments, which are formed in the most varied plant organs under the most differing conditions, involves difficulties of no mean order. It may be hoped that new investigations, especially quantitative ones, will enable us to obtain deeper insight into the physiology of these pigments.

The especially interesting natural alterations in the colour of flower petals, both in the temperate as well as in the tropic zones, are still not clear from the physiological and cytological point of view. They represent together with the artificial alterations of the flower colour pattern, as initiated by R. Harder, a field in which there are many opportunities for interesting investigations.

The greater variations of anthocyanins in flowers and fruits indicate that the colour of flowers and fruits is manifestly of some importance for the attraction of insects and birds, and that by means of natural selection a greater variation of anthocyanins has developed in these organs.

The study of heredity with respect to anthocyanins has achieved manifest success. The special value of these researches resides in the fact that not only the colour as a whole was followed in the process of heredity, but that also the inheritance of chemical individual substances was studied. This led to the winning of important evidence concerning the biosynthesis of the anthocyanins.

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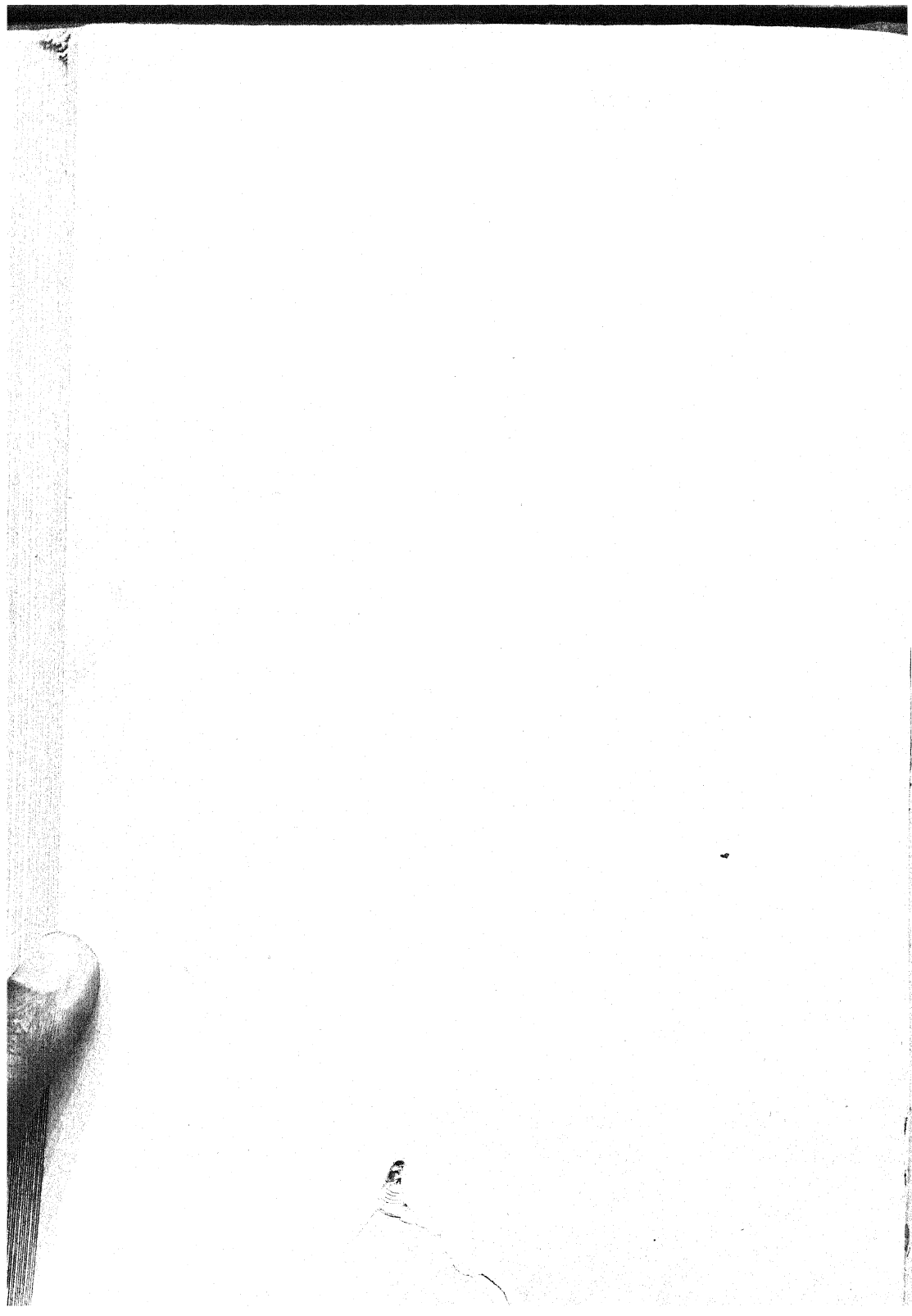
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CYTOLOGY AND GENETICS OF FORAGE GRASSES¹

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INTRODUCTION

A large proportion of the species of Gramineae have been used at one time or another for forage. Certain of these, the tame hay and pasture grasses, are grown almost exclusively for this purpose. The millets and sorghums are important as human food in some regions, particularly in Asia, but these grasses are grown fairly extensively in the United States as temporary hay and pasture or silage crops. The cereals (wheat, oats, barley, corn), although cultivated primarily for grain, are used also for grazing, hay or silage in some instances. In addition to the cultivated grasses, a large number of species make up the native pastures—the range of the western United States and similar native grassland areas elsewhere in the world. Some of these species, included in the genera *Andropogon*, *Agropyron*, *Bouteloua*, *Stipa* and others, occur abundantly and constitute an important part of the herbage. Others occur sporadically and are important for grazing only in limited areas or for a short time during the growing season. In recent years, with the increased attention given to maintenance and reestablishment of the ranges, the more important wild species have been seeded on extensive areas. Thus some of these wild species are becoming cultivated grasses.

Cytological and genetical investigations of the grasses have been initiated primarily for two reasons, first, to serve as an adjunct to morphological data in studies of the taxonomy and phylogeny of the Gramineae, and second, to provide fundamental information for the improvement of species by breeding. In relation to the

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systematic and phylogenetic studies, chromosome numbers of numerous species have been determined, polyploidy and intraspecific chromosome races have been discovered in many genera and species, and several interspecific and intergeneric hybrids have been investigated. Increased appreciation of the importance of forage plants, which led to the initiation of many grass breeding projects during the last two decades, added a great stimulus to cytological and genetical investigations of the more important species. These investigations include studies of meiotic behavior within species and in interspecific hybrids, origin of polyploidy, cytogenetics of polyploids, inheritance, and linkage relations.

Although the cereal grasses—*Triticum*, *Avena*, *Hordeum*, *Secale*, *Oryza*, *Zea*—are used, in some cases extensively, for forage, they are chiefly of importance for their grain. The genus *Aegilops* is of interest primarily because of its phylogenetic relation to *Triticum*. Literature dealing with these genera will be included in the present review only when the results seem to have a direct bearing upon the cytology and genetics of the forage grasses.

KARYOTYPE ANALYSIS AND PHYLOGENY

The value of cytological investigation as an aid in establishing systematic and phylogenetic relationships among species and genera was recognized by many (*e.g.*, 240, 241). Hunter (153) pointed out that, in respect to taxonomic significance of karyotypic peculiarities, first place is taken by basic number and size of chromosomes. Next in order of importance come idiogram types, characterized by changes in length and structure of the arms of individual chromosomes. Limitations of karyotype analysis in systematics have been emphasized (*e.g.*, 24). Morphologically similar chromosomes in diverse species may not be homologous. Furthermore, variations in chromosome shape and size may occur in strains of the same species; the latter character particularly is known to be under genotypic control (101, 542). Despite these limitations, chromosome numbers, size and morphology provide critical information regarding phylogenetic relationships when used in conjunction with morphological, geographical and ecological studies (*cf.* 24).

Several investigators (*cf.* 21, 153) have attempted to construct a natural system of classification of the Gramineae. Consideration of these systems is not within the scope of this review. The first

large-scale attempt to use cytological information as an aid in classification of the grasses was that of Avdulov (21), but other extensive investigations of this kind have also been reported (71-74, 120, 153, 224, 516, 525). In addition, detailed determinations of chromosome numbers have been given for species in the tribes Festuceae (77), Phalarideae (269, 359), Oryzeae (376) and Paniceae (60), in the genera *Bromus* (100), *Festuca* (242), *Poa* (18, 52, and others), *Glyceria* (75, 76), *Agropyron* (364, 365, 502), *Hordeum* (14, 135, 363, 541), *Deschampsia* and *Aira* (138), *Danthonia* (63), *Agrostis* (511), *Alopecurus* (188, 534), *Bouteloua* (124), *Paspalum* (59, 487) and *Sorghum* (128, 157, 198, 253, 443), and in miscellaneous species (202, 268, 313 to 316, 326, 330, 481, 490, 512, 524). Determinations of one or a few species are found in many reports. Although the authors, in most instances, did not deal with the general problems of phylogeny and systematics, the chromosome numbers reported are invaluable in presenting a more complete picture of the relationships among species, genera, tribes and subfamilies.

From his extensive investigations of chromosome numbers, size and morphology in the grasses, Avdulov (21) attempted to construct a more natural system of classification. Karyological data were correlated with morphology of flowers, shape of first leaf, anatomy of leaves, structure of starch grains, and ecological distribution. On the basis of these data, he divided the Gramineae into two sub-families, the Sacchariferae and Poateae. The latter was further subdivided into Phragmitiformes and Festuciformes. The Sacchariferae have small chromosomes in multiples of 9 or 10, the Phragmitiformes have small chromosomes in multiples of 12 (this group has many features characteristic of a primitive type), and the Festuciformes have large chromosomes in multiples of 7 or less. Cytologically, evolution has proceeded, according to Avdulov (21), in the first stage by a reduction in number and an increase in size of chromosomes. The decrease in number finally continued without a corresponding increase in size to genera with 6 and 5 as the basic number. This procedure is more true of the Festuciformes which have passed into the colder regions of the world. The Sacchariferae have remained in the tropics, and the chromosome number has not fallen below 9. The final stages of evolution have involved also changes in disposition of the chromatin in the chromosomes, i.e.,

in chromosome morphology. Avdulov (21) agrees with Levitskii (239, 240, 241) in considering the more symmetrical chromosomes as more primitive, the derived types having unequal length of arm. This view has been substantiated by findings in *Allium* (236), *Vicia* (491) and *Crepis* (24).

Hunter (153) proposed minor modifications to Avdulov's classification, but, in general, his results supported Avdulov's conclusions. As already pointed out (153, 525), more complete cytological information is required for a number of tribes before an accurate evaluation of the systematics and phylogeny of the Gramineae can be given.

CHROMOSOME NUMBERS

The chromosome numbers of numerous species have been reported in recent years, making available for summary a more extensive list than has been compiled previously (125 to 127, 267, 550). In Table 1, 805 species in 142 genera are recorded. These do not include species of *Triticum*, *Aegilops*, *Secale*, *Avena* or *Zea*. The arrangement of tribes and genera follows Hitchcock (150), and the species are listed, so far as possible, in ascending order of chromosome number.

In 360 species the somatic numbers are multiples of 7. Multiples of 5 occur less frequently (163 species), followed in order by 6(85), 9(82), 8(7), 13(7), 17(5) and 11(3). In compiling these data, species with multiples of 10 were counted as multiples of 5, since it was impossible in many species to determine from chromosome numbers alone whether the basic number was 5 or 10. Similarly, some species included among those with $x=6$ have numbers that are multiples of 12. Species with $2n=36$ might be hexaploids ($x=6$) or tetraploids ($x=9$). In nine cases it was impossible to determine from related species whether $x=6$ or 9. Variable chromosome numbers were reported in 16 species, nine of *Poa*, three of *Bouteloua*, two of *Alopecurus*, one of *Deschampsia* and one *Saccharum*. Apomixis occurs in some of the *Poa* species, thus accounting for perpetuation of variable aneuploid numbers. In *Alopecurus*, variability may be attributable to the high degree of polyploidy (120).

The basic number could not be determined in 56 additional species, including 39 of *Stipa* in which somatic numbers of 24, 28, 32,

34, 36, 40, 42, 44, 46, 48, 64, 66, 68, 70 and 82 are known. In each of five *Glyceria* species and, similarly, in several others, multiples of 7 and of 10 have been reported by different authors. It seems probable in such instances that the species was incorrectly identified by one of the authors or that the count was in error. In a few cases, numbers differing by two to four chromosomes were reported, the discrepancy resulting probably from errors in counting or the occurrence of aneuploids.

A basic number of 12 was postulated for the Bambuseae (21, 596). Since species with $2n = 54$ occur, the basic number may be 6, however, instead of 12. A majority of species in Festuceae, Hordeae, Aveneae and Agrostideae have chromosome numbers in multiples of 7. Exceptions occur in each tribe, however. *Phragmites*, *Schismus* and *Oryzopsis* have $x = 6$ or 12, *Sporobolus* and *Melica* have $x = 9$, *Eragrostis* and *Aleuropsis* have $x = 10$, and *Dupontia* and *Aristida* have $x = 11$. In *Pleuropogon*, the three species reported have $x = 8(77)$, although one collection of *P. californicus* had $2n = 14$. Variations in basic number occur also among species in some genera. *Danthonia*, *Triodia* and *Trisetum* have species with $x = 6$ and 7; *Milium*, $x = 7$ and 9; *Briza* and *Glyceria*, $x = 7$ and 10; *Koeleria* and *Deschampsia*, $x = 7$ and 13; *Lepturus*, $x = 7, 9$ (or 6) and 13; *Orcuttia*, $x = 6$ (or 12), 8 and 13; and *Muhlenbergia* and *Sporobolus*, $x = 9$ and 10. The extensive aneuploid series in *Stipa* has been noted above.

Chromosome numbers of too few species of *Zoysieae*, *Chlorideae*, *Phalarideae*, *Zizaneae* and *Melinideae* are known to indicate the predominant basic number in these tribes. In the *Oryzeae*, $x = 12$ (or 6) in all species reported except *Lygeum spartum* ($2n = 40$).

Basic numbers of 9 and 10 (or 5) predominate in the *Paniceae*, *Andropogoneae* and *Maydeae*. Four species of *Pennisetum* have $x = 7$, while $x = 6$ (or 12) occurs in some species of *Paspalum*, the two species of *Arthraxon* and in *Tristachya hispida*. Aneuploid series, similar to but less extensive than that found in *Stipa*, occur in *Digitaria*, two species having $x = 8$, an uncommon basic number in the *Gramineae*. Three species of *Echinochloa* have $x = 17$.

In consideration of the great preponderance of species with chromosome numbers in multiples of 7, it might seem logical to conclude, as Wanscher (584) did, that 7 is the primary basic number of the *Gramineae*. A difficulty, however, in drawing con-

clusions from the high frequency of species with $x=7$ is that chromosome numbers have not yet been determined for a large proportion of grass species. In Table 1, chromosome numbers are given for species in 142 genera, whereas Hitchcock (150) lists 159 genera of grasses in the United States alone. Furthermore, 7 occurs as a basic number more commonly in the Festuceae, Hordeae, Aveneae and Agrostideae that have their distributions mainly in the temperate and cold regions of the northern hemisphere. Species and genera of these tribes have, in general, been investigated more extensively than those of the tropics and southern hemisphere.

Other evidence has been offered also to support the assumption of 7 as the primary basic number. Huskins and Smith (158) concluded from occurrence of quadrivalents at meiosis in *Sorghum* species and from the existence of duplicate genes in *Zea mays* that these species with $2n = 20$ were polyploid, probably with a primary basic number of 7. Powers and Clark (373) arrived at the same conclusion from a statistical study of pairing at diakinesis in partially asynaptic *Zea mays*. The variability and frequency distribution of paired and unpaired chromosomes were in agreement with the hypothesis that the ten pairs of chromosomes responded as seven independent units.

Five also has been proposed as the primary basic number (120, 130, 317, 362). Five is the lowest basic number found in the Gramineae and seemed, therefore, to be the logical primary basic number (120). Evidence considered (120) to support this hypothesis is secondary association in units of five at metaphase I and II, occurrence of quadrivalents in supposedly diploid species, and bivalent formation in haploids. Secondary pairing of bivalents in five groups was reported in *Oryza sativa* (262, 484) and *Puccinellia vahliana* (120). In the latter case, the two bivalents in each associated group were morphologically similar.

Quadrivalents at diakinesis were reported in *Oryza sativa* (484), and a single quadrivalent has been found in certain plants of the diploid ($2n = 14$) species, *Puccinellia vahliana* (120), *Festuca pratensis* (377), *Dactylis aschersoniana* (281) and *Briza media* (204). Flovik (120) regarded quadrivalents in the diploid species as evidence that 7 was a derived number, presumably from 5. The case of *Briza media* was considered (120) particularly significant because of the existence of *B. minor* with $2n = 10$. On the other hand, some

workers have attributed the quadrivalents to structural hybridity (204, 281, 377). Kattermann's (204, 205, 206) results with *B. media* seem particularly conclusive in that regard.

Bivalents were observed occasionally at metaphase I of meiosis in haploid plants of *Secale cereale* (238, 348), *Triticum monococcum* (cf. 120, 159) and *Hordeum distichum* (552). This has been interpreted (120) as supporting the hypothesis that $x=7$ is a derived number. A similar suggestion was offered by Tometrop (552). On the contrary, Levan (238) did not consider bivalent formation in haploid *Secale cereale* as evidence of secondary diploidy derived from $x=5$. In contrast to haploids, no evidence from metaphase pairing was found in triploid *Lolium perenne* (300) of homology between chromosomes of the haploid set.

Species with $2n=10$ occur in *Briza*, *Anthoxanthum* and *Sorghum*. Flovik (120) considered that species with $x=10$ were derived from these primary types. Multivalents at meiosis in *Sorghum vulgare* (158) may indicate that this species is a tetraploid instead of a secondary polyploid, as suggested by the authors (158). On the other hand, Garber (128) did not find multivalents in *Sorghum* spp. with $2n=20$ and questioned the derivation of the *Eu-sorghum* ($2n=20$) from the *Para-sorghum* ($2n=10$) group. A related species of the Andropogoneae, *Hyparrhenia hirta*, has $2n=30$, however, supporting the assumption of $x=5$ in this tribe (128). Likewise, 15 bivalents are formed in *Narenga porphyrocoma* (166).

Gates (130) and his students, particularly Pathak (362), consider that the number of chromosomes of the haploid set with nucleolus organizers has a bearing on the degree of polyploidy involved. The occurrence of two such chromosomes in some grasses with $2n=14$ was interpreted as an indication of polyploidy in these forms, thus supporting the assumption of 5 as the primary basic number (130, 362). The unreliability of number of nucleolar chromosomes in determining the existence of polyploidy has been pointed out (31, 300).

Avdulov's (21) conclusion that 12 is the primary basic number was based largely on his assumption that more primitive forms, *Bambusa*, *Oryza*, *Ehrharta* and *Phragmites*, have numbers in multiples of 12, whereas some of the more specialized and recently derived groups have numbers in multiples of 5, 6 or 7. Yamaura (596) also considered 12 the basic number in the Bambuseae, but

Hunter (153) questioned this because of the occurrence of species with $2n = 54$. Evidence of polyploidy has been reported in *Oryza sativa* (272, 317, 358, 484) and *Ehrharta* spp. (359), suggesting that 12 may not be the primary basic number. Contrary to the assumption of polyploidy in *Oryza sativa*, bivalents occur rarely or not at all in interspecific hybrids involving this species (149, 318). Furthermore, no species are known in the Oryzeae with any number other than $x = 12$ (except *Lygeum spartum*— $2n = 40$). *Phragmites communis* has been reported with 36, 48 and about 96 chromosomes (Table 1), suggesting a basic number of 6. In this regard it is noteworthy that *Phragmites* may be one of the most primitive grasses; fossil representatives belonging to the genus are known from the middle Tertiary Period and especially from Myocene deposits (120). Six has been found also as the basic number of some species of *Danthonia* which are among the most primitive of the Aveneae (63).

Examination of chromosome numbers in conjunction with evidence available at present on phylogenetic relationships in the Gramineae does not seem to provide critical evidence regarding the primary basic number. The data on chromosome numbers are, in themselves, incomplete, and addition of new data may alter appreciably the frequency of various basic numbers. Furthermore, inaccuracies no doubt exist both in the identification of certain species and in determination of chromosome number. An instance of the first difficulty is found in Church's (74) discussion of Avdulov's (21) data for species of *Spartina*. In order that the cytological analysis be more nearly complete, there is need for supplementing the data on chromosome numbers with information on chromosome morphology, meiotic behavior in species and interspecific hybrids, genetical relationships, and methods of reproduction. Finally, these cytological data must be correlated with morphological, ecological, geographical and paleontological data in order that a more natural phylogenetic system may be devised.

CHROMOSOME MORPHOLOGY

Differences observed in chromosome morphology include size, shape (relative length of the two arms determined by position of the centromere) and presence of secondary constrictions and satellites. Avdulov (21) reported that species included in his Sac-

chariferae and Phragmitiformes had small chromosomes, while species of Festuciformes had large chromosomes. Similar observations have revealed that chromosomes of the Maydeae generally are larger than those of the Andropogoneae (153). In addition to the general size differences exhibited by larger groups, considerable variation has been observed among species of the same genus. *Pennisetum glaucum* has larger chromosomes than is known for any other species of Paniceae (153), the difference, in comparison with *P. purpureum*, being great enough to permit identification of the parental chromosomes in F_1 hybrids (62). Average lengths of chromosomes of *Sorghum versicolor* ($2n = 10$), *S. vulgare* ($2n = 20$) and *S. halepense* ($2n = 40$) were 4.86, 2.24 and 1.98 microns, respectively (198). The much greater chromosome length in the Para-sorghum ($2n = 10$) compared with the Eusorghum group ($2n = 20$) was noted also by Garber (128). In *Glyceria*, species with $x = 7$ have large chromosomes similar to those of *Festuca* and of other 7-chromosome grasses, whereas species with $x = 10$ have small chromosomes (75). Nielsen (326) reported variations in length and diameter of chromosomes in root tips of several species. Similar differences were reported for species of *Spartina* and *Andropogon* (75) and for several species of arctic grasses (120). Ghimpu (131) found that the cultivated barleys had a greater total volume of chromosomes than wild species with $2n = 14$. Variations among different plants of the same species have been observed in *Bouteloua* spp. (124), *Lolium perenne* (312, 542) and *Dactylis glomerata* (312).

For use in cytogenetic studies, morphological identification of the individual chromosomes of the genome provides an important tool. Differences in size among the chromosomes within species have been reported for *Lolium* spp. (116), *Melica* spp. (525), *Bromus carinatus* (526), *Sorghum* spp. (128, 157, 163, 198), *Spartina* spp. and *Andropogon* spp. (74), *Festuca elatior* var. *pratensis* (312, 377), *Alopecurus pratensis* (377), *Setaria* spp. (225), *Anthoxanthum odoratum* (359), *Phalaris* spp. (269), several species of arctic grasses (120), *Dactylis glomerata*, *Lolium perenne*, *Phleum pratense* (312) and several other species (326). It is probable that careful analysis would reveal differences within genomes in most species of grasses.

Because of variations among plants of the same species and the

influence of fixatives and other conditions on chromosome length, a more critical diagnostic character is chromosome shape, determined by position of the centromere. Variations in this character among chromosomes of the same species have been reported by several investigators (21, 74, 120, 128, 153, 163, 269, 312, 326, 377, 525).

In addition to the primary constriction (centromere), secondary constrictions or satellites form a characteristic morphological feature of certain chromosomes in mitosis. The relationship of secondary constrictions and satellites to the nucleolus was recognized by Heitz (145). Later McClintock (259) demonstrated in maize that nucleolus organization was controlled by a specialized part of the chromosome, the nucleolus organizer. On the basis of these investigations it may be assumed that in most plants two or more chromosomes will have secondary constrictions or satellites in mitotic divisions. Satellites have been observed in *Melica* spp. (525), *Phleum* spp. (292), *Phalaris canariensis* (269), *P. brachystachys* (269) and *Sorghum purpureo-sericeum* (163). Secondary constrictions occur in *Agropyron junceum* (503), *Calamagrostis epigeios* (326), *Stipa pulchra* (326), *Anthoxanthum odoratum* (359) and *Lolium perenne* (300). Both satellites and secondary constrictions have been reported in *Agropyron spicatum* (365), *Muhlenbergia pungens* (326) and *Dactylis glomerata* (312).

In most plants a secondary constriction has been reported in only one or two chromosomes in each genome. In contrast, Flovik (120) reported that "all species have secondary constrictions in all or mostly all chromosomes and frequently in an extremely high number, as for instance in *Phippsia* and *Puccinellia*". In some species of these genera, certain chromosomes had as many as three or four secondary constrictions. Similar observations were reported for *Festuca pratensis* and *Alopecurus pratensis* (377). Failure of other investigators to observe the high frequency of constrictions was attributed (120) to the fixing solution used. In most studies of root tip mitoses the primary objective has been determination of chromosome number. Fixation most favorable for chromosome counting frequently is least suitable for determination of constrictions. It seems probable, however, that the numerous constrictions observed (120) are of a kind different from the secondary constrictions reported by others.

The classical investigations on morphology of mid-prophase chromosomes of *Zea mays* (257) provided an invaluable tool for cytogenetic studies of that species (473 and many subsequent investigations). Preliminary investigations with some species (*Lolium perenne*, *Sorghum vulgare*, for example) indicate that studies of mid-prophase chromosomes will be much more difficult in many other grasses than in maize. Pycnotic knobs in characteristic positions serve as valuable markers in identification of certain individual chromosomes of maize. Such knobs have not been observed in *L. perenne* (312) or *S. vulgare* (254). In *Phleum pratense* ($2n = 42$) three chromosome pairs, presumably one from each genome, have a single pycnotic knob (292).

CHROMOSOME FRAGMENTS

Centric chromosome fragments have been reported in several species—*Alopecurus alpinus*, *Dupontia fisheri*, *D. fisheri* var. *psilosantha*, *Poa alpigena*, *P. alpigena* var. *vivipara* and *P. alpina* var. *vivipara* (120), *Briza elatior* (22), *Paspalum stolonifera* (23), *Anthoxanthum odoratum* (153), *A. aristatum* (355), *Agrostis trinii* (511), *Sorghum verticilliflorum* (158), *S. purpureo-sericeum* (163, 164), *Festuca elatior* var. *pratensis* (312, 377), *Alopecurus pratensis* (377), *Dactylis glomerata* (377) and *D. aschersoniana* (281). None of these cases has been investigated as thoroughly as the B chromosomes of *Zea mays* (380), but it has been presumed that the fragments may be analogous to the B chromosomes, at least in some cases.

Avdulov and Titova (23) suggested that fragments found in a plant of *Paspalum stolonifera* probably were devoid of genetic material, impoverishment of the chromosomes representing a step in the process of decrease in chromosome number. In *Anthoxanthum odoratum* the fragments (two pairs of large and one pair of small almost spherical fragments) were less deeply stained than the remainder of the chromosomes (153).

The supernumerary chromosomes of *Sorghum purpureo-sericeum* have been studied more intensively than others reported in the forage grasses. Janaki Ammal (163) considered them morphologically identical with the shortest chromosomes of the normal set. At meiosis the extra chromosomes paired with one another, forming bivalents, trivalents and quadrivalents. Janaki Ammal

(163, 164) considered these chromosomes analogous to the B chromosomes of maize and suggested that 5-chromosome sorghums arose from a basic number of 7, the extra chromosomes representing relics of that process. Darlington and Thomas (104) found in *S. purpureo-sericeum* several types of B chromosomes that paired in meiosis when homologous. A very anomalous behavior has been reported for these chromosomes. B chromosomes were found in the microsporocytes of 40 of the 100 plants examined (164). Root tips were examined from 20 of the plants with B chromosomes in pollen mother cells, and in each case only $2n = 10$ was found. A more nearly complete developmental study revealed that the B chromosomes were lost in the radicle before seed ripening (104). In shoot tissue they were lost as the plants reached maturity but persisted in the ovaries and anthers. The B chromosomes were lost in mitosis by their failure to become oriented on the metaphase plate.

The fragments in *Festuca elatior* usually paired with one another or occurred as univalents, but rarely was a fragment paired with a normal chromosome (312, 377). Similar behavior was found also for the fragments of *Alopecurus pratensis* (377).

In hybrids of *Lolium perenne* \times *Festuca arundinacea* one F_1 plant and three plants from the backcross to the male parent had an extra centric fragment about one-half as long as the normal chromosomes (366). The origin of such centric fragments may perhaps be accounted for by misdivision of the centromere (102, 472).

The fragment chromosomes may represent, as has been suggested (23, 163), a stage in the evolutionary process of reduction in chromosome number. On the other hand, they may provide, because of their centromere, a ready mechanism for increase in basic number. More intensive studies of the origin and behavior of centric fragments in the forage grasses are needed.

POLYPLOIDY

Occurrence

Polyploidy occurs frequently in most families of the angiosperms, particularly in the Gramineae, Polygonaceae, Nymphaeaceae, Rosaceae and Malvaceae (521). Among the species and chromosome races summarized in Table 1, more than two-thirds are polyploid or have one or more polyploid races. Polyploid species or chromo-

some races are found in almost every genus, the two principal exceptions being *Melica* (524, 525) and *Lolium*.

A striking feature of polyploidy in the Gramineae is the occurrence, within species, of races differing in chromosome number. Müntzing (279) has considered this phenomenon in detail. In 1936 he (279) estimated that the number of cases of intraspecific chromosome races known in the plant kingdom was something over one hundred. In Table 1 there are recorded 99 species with chromosome races, not including *Poa pratensis* and others in which the persistence of aneuploid forms is attributable to apomixis. Included among the species with intraspecific chromosome races are several of the important forage grasses. An extreme example of polyploid races within species is *Panicum virgatum*. In 59 collections, Nielsen (328) found $2n$ numbers of 18, 36, 54, 72, 90 and 108 chromosomes ($2x$, $4x$, $6x$, $8x$, $10x$ and $12x$). Among 17 collections (isolates) obtained from an area of not more than ten acres near Chippewa Falls, Wisconsin, $2x$, $4x$, $6x$, $8x$ and $10x$ forms were found.

Chromosome races occur in some species known to be highly variable. *Festuca elatior*, for example, is separated taxonomically into several varieties (242, 516) that have been given specific rank by some authorities. In other species, such as *Bromus inermis*, taxonomic varieties are not recognized (150). Müntzing (279) concluded that intraspecific chromosome races are always more or less different morphologically, but Nielsen's (328) results were contrary. In an intensive study of variation in *Panicum virgatum*, Nielsen (328) measured several characters that might prove useful in the morphological separation of the chromosome races. Considering all possible comparisons among isolates with different chromosome numbers, 66.4% showed statistically significant differences, while among isolates of the same chromosome numbers, 58.6% of the differences were significant. Thus differences were found among isolates of the same chromosome number almost as frequently as among those from different chromosome races.

Types of Polyploids

Cytologists have agreed generally upon classification of polyploids as autopolyploids and allopolyploids. This classification may be based either upon origin of the polyploid or upon degree of

differentiation of the genomes of the polyploid. On the former basis, autopolyploid refers to derivatives of chromosome doubling within a fertile species, whereas allopolyploid refers to derivatives of doubling in interspecific hybrids. On the second basis of classification, the autopolyploid has three or more genomes that are structurally identical (although they may be genetically different), while the allopolyploid has two or more different genomes. Neither system of classification is entirely satisfactory, the basic difficulty being that polyploids are not differentiated naturally into two discrete classes. At one extreme there may be polyploids derived by repeated chromosome doubling from the haploid (247), while at the other extreme are types derived from species hybrids in which chromosome pairing does not occur (193). Most naturally occurring polyploids probably occupy an intermediate position with regard to chromosomal differentiation.

There is no universal agreement on what constitutes a valid species, and the sterility of species hybrids varies within wide limits. Furthermore, in the analysis of established species it is impossible in many cases to postulate with certainty the mode of origin, whether by doubling of the fertile species or by doubling in the hybrid between two closely related species in which chromosomal differentiation has not proceeded very far. From the standpoint of application to existing polyploids a classification based upon differential affinity of the chromosomes of different genomes seems most appropriate, for it is this characteristic that is basic to the cytogenetical behavior of the species. For the purposes of this classification, however, observations of multivalent frequency in meiosis will be inadequate. There will be required a careful cytological and genetical analysis of the species and, in many cases, of species hybrids, polyploids and nullo- and polysomics.

Information on type of polyploidy is available for relatively few forage grasses, the most completely investigated being *Dactylis glomerata* and *Phleum pratense*. In *D. glomerata*, Müntzing (275, 281) reported the regular occurrence of quadrivalents in meiosis, the maximum number of 7 having been observed in some sporocytes. In F_1 of *D. glomerata* \times *D. aschersoniana* meiotic behavior was of the type expected in an autotriploid (281). On the basis of these cytological data he (275, 281) concluded that *D. glomerata* is an autotetraploid derived by chromosome doubling from *D. ascher-*

soniana. Confirmatory evidence was provided by studies of polyploid plants ($2n = 14$) of *D. glomerata* (285). These plants resembled *D. ascherssoniana* morphologically and had a regular meiosis with seven bivalents. The above reports (281) of meiotic behavior in *D. glomerata* have been substantiated (296, 297, 307–310). Furthermore, the assumption of autopolyploid behavior has been verified genetically (290, 293, 304).

Phleum pratense was originally considered an allohexaploid (278). From chromosome doubling in the hybrid *P. pratense* ($2n = 14$) \times *P. alpinum* ($2n = 28$), fertile hexaploid plants were obtained that produced fertile offspring when crossed with hexaploid *P. pratense* (132, 134). This was one of the early instances of synthesis by hybridization and chromosome doubling of an existing polyploid species. Additional cytological evidence supporting the hypothesis of allohexaploidy was the reported absence of multivalents at metaphase I (278, 287, 349). There were rather limited genetical data available. Typical monohybrid ratios were reported for resistance to rust (25, 78, 306). Clarke (78) explained the occurrence of chlorophyll-deficient seedlings among inbred progenies by the assumption of triplicate factors. In similar material Wexelsen (587) obtained results interpreted on the basis of single, duplicate and triplicate factors, assuming that the species was an allohexaploid. In two families, however, the results suggested the possibility of tetrasomic inheritance.

In contrast with the cytological results previously obtained, 14 bivalents were found (347) in F_1 of *Phleum pratense* ($2n = 14$) \times *P. pratense* ($2n = 42$), 28 bivalents were found (287) in 63-chromosome timothy plants obtained from twin seedlings, and a maximum of 12 bivalents were observed (292) in F_1 of *P. pratense* ($2n = 42$) \times *P. subulatum* ($2n = 14$). In polyploid ($2n = 21$) plants obtained from twin seedlings there was variable pairing from seven bivalents plus seven univalents to 21 univalents, the former being the most common condition (237, 349). In addition, occasional trivalents were observed. In *P. pratense* ($2n = 14$) \times *P. alpinum* ($2n = 28$), the frequency of trivalents, bivalents and univalents was similar to that found in autotriploid *P. pratense* ($2n = 14$). Diploid *P. pratense* and tetraploid *P. alpinum* are the putative parents of hexaploid *P. pratense* (132, 134). Thus, the

results (349) suggest homology between chromosomes of the genomes of *P. pratense* ($2n = 42$).

Consistent with these results, further studies (302) of meiosis in plants of hexaploid *Phleum pratense* revealed a frequency of quadrivalents ranging from 0 to 7 per sporocyte, the average being 2.9 to 4.9 for the seven plants. Also sexivalents occurred in about one-third of the sporocytes, the frequency varying from 1 to 3. Genetic data (302) from first and second generation inbred progenies of five unrelated plants confirmed the cytological evidence that this species is not an allohexaploid. It was impossible from the cytological and genetical data available to determine whether there were six homologous genomes or four genomes of one kind and two of another.

On the basis of the regular and frequent occurrence of quadrivalents in meiosis, autopolyploidy has been postulated for *Arrhenatherum elatius* (203, 307, 308), tetraploid *Agropyron cristatum* (307, 308), *Anthoxanthum odoratum* (203, 355, 359), *Hordeum bulbosum* (31, 70) and *Poa palustris* (212). In *Agropyron cristatum* and *Anthoxanthum odoratum* there are related diploid forms from which the tetraploid races presumably arose by chromosome doubling. No critical genetical data have been reported for any of these species.

Other reports of autopolyploidy, based, however, upon rather meager evidence, include *Eleusine coracana* (224), *Phippsia algida*, *P. concinna*, *Puccinellia angustata*, *Dupontia fisheri*, *Trisetum spicatum* (120), *Festuca ovina* (558), *Briza media* and *Agrostis trinii* (478). Müntzing (279) considered that most of the polyploid intraspecific chromosome races were autopolyploid, and developed several lines of evidence to support his assumption. Contrary to the report (224), based upon limited cytological evidence, of autopolyploidy in *Eleusine coracana*, the genetic ratios (387-390, 397, 398, 400-404, 580) agree more closely with those expected in an allopolyploid.

In the 72 chromosome race of *Tripsacum dactyloides*, 6_{IV} were formed commonly in meiosis, whereas 18_{II} were always found in the 36-chromosome race. From these results Anderson (12) concluded that the latter race is an allopolyploid with two highly differentiated genomes. The 72-chromosome race arose from

chromosome doubling in the hybrid of this race with another allotetraploid with one common genome.

Multivalents have been observed at meiosis in a few other polyploid species. The occurrence of quadrivalents and sexivalents at meiosis in *Sorghum vulgare* and of quadrivalents, sexivalents and octavalents in *S. halepense* has been reported (158). Occasional quadrivalents and other multivalents were found in *Brachypodium pinnatum* (202), *Bromus erectus* var. *eu-erectus* (203), *Alopecurus alpinus*, *Calamagrostis neglecta*, *Festuca rubra* and *Puccinellia phryganodes* (120), *Phalaris minor* and *Ehrharta calycina* (359), *Agropyron junceum* and *A. repens* (352), *Festuca elatior* var. *arundinacea* (312), *Agropyron glaucum* and *A. elongatum* (367), and *Lygeum spartum* (376). Multivalents were not observed at meiosis in *Bromus inermis* (217), *B. mollis* (219) and *Alopecurus pratensis* (377).

More critical cytological and genetical studies are needed in many species before a reliable evaluation of the incidence of auto- and allopolyploidy can be made. These data are not available at present, even for a majority of the most important forage grasses.

On the assumption of monophyletic origin of the Gramineae, a large part of the species must be secondary diploids and polyploids. Evidence for secondary polyploidy usually is based on the occurrence of secondary association at metaphase of meiosis which is presumed to result from relic homologies (235). This hypothesis has been criticized (144) and discussed (68). Secondary association has been observed in several grass species (120, 317, 359, 484).

Characteristics of Natural Polyploids

Polyploids are known to differ morphologically and physiologically from related diploids. These differences have been summarized by others (36, 101, 279, 521). In general, they may be attributed, to a considerable extent, to the larger nucleus and cell resulting from chromosome doubling. Differences due to recombination of genes from different species (in allopolyploids) would not be constant features of polyploids in general. Stählin (516) reported that with an increase in chromosome number in the grasses from diploidy to tetra- and hexaploidy there was generally an increase in plant size and organ size. With further increase in

chromosome number to octo- or decaploidy there was no further increase in plant size; there was in some instances, in fact, a decrease. Müntzing (279) likewise postulated a maximum favorable chromosome number (which differs from one genus to another) above which there was no beneficial effect of increased numbers. In *Dactylis*, pentaploid derivatives of *D. aschersoniana* \times *D. glomerata* may be more vigorous than the tetraploid, but an octoploid ($2n = 56$) plant was dwarf and could not be kept alive (281).

Hagerup (137) was one of the first to recognize the relationship of polyploidy in plants to their ecological adaptation and geographical distribution. In this connection he stated that polyploids have acquired new genetical and morphological characters whereby they are enabled to grow in other localities, so they can have a new ecological and phyto-geographical value. Among the flora of Timbuktu, one grass genus was studied. *Eragrostis cambessediana* ($2n = 20$) was annual and limited to the seashore where soil and atmosphere were always moist. *E. albida* ($2n = 40$) was very similar but was perennial and occurred in places of intermediate moisture, while *E. pallescens* ($2n = 80$) was a complete xerophyte. Later, Hagerup (138) reported that the tetraploid and octoploid species of *Deschampsia* have a wider ecological and geographical range than the diploid. Similar results were obtained by Tischler (551). In the flora of Schleswig-Holstein, among the species of which the chromosome numbers are known, 44% are polyploid. The frequency of polyploids varied, however, from 60% in the north to 27% in the south. The percentage of polyploidy is higher in the Faroe Islands but lower in Sicily than in Schleswig-Holstein. Studies of the flora of Schleswig-Holstein were extended by Rohweder (477). In the ditches of certain marshes flooded daily with the lime-bearing tidal waters of the Elbe, 95% of the species were polyploid. Likewise, in the Great Wapelfeld Moor with marl sub-soil, 79% of the species were polyploid, while in more recent land formations, the Island of Amrum, only 38% of polyploids are found.

In the high mountain regions of Pamir and Altai about 150 and 200 species, respectively, mostly Gramineae, were studied. Of these, 85% and 65%, respectively, were polyploids (512). The genera *Poa* and *Alopecurus* were represented in the highlands by polyploids (*P. alpina*, *P. altaica*, *P. tibetica*, *A. vaginatus*, *A.*

mucronatus, etc.), but migration into the highlands was not always concomitant with increased polyploidy. Apparently migration to the north usually was accompanied also by increased incidence of polyploidy. A similar correlation between high polyploidy and extremes of climate was observed in *Alopecurus* (534) and *Agrostis* (511). In the latter genus, the polyploid species occur on the eastern and northern limits of the range of distribution and in alpine regions.

Most of the arctic grasses studied by Flovik (120) were polyploids, some (*Alopecurus alpinus*— $2n = 112$ and 114) with very high chromosome numbers. Among 68 species and varieties from Spitzbergen (some of them grasses), 80% were polyploids (121). The only diploid grass found was *Puccinellia vahliana* ($2n = 14$). This represented an exception to the general rule that polyploid species had a more northern distribution; polyploid species of *Puccinellia* occur to the south. Increase in percentage of polyploids with increased latitude has been reported also by Löve and Löve (255). A high frequency of polyploids was found among the forage grasses of California where tolerance to heat and drouth is essential for survival (525). Polyploidy and tolerance were correlated. For example, five grasses were collected in the Mohave Desert. Three of these, *Poa secunda*, *Elymus condensatus* and *Sporobolus airoides*, are high polyploids; *Oryzopsis hymenoides* is the only polyploid yet known in the genus, while *Stipa speciosa* has one of the higher chromosome numbers of its genus (525).

In addition to variations in distribution and adaptation between diploid and polyploid species, there have been reported also numerous instances of differences among intraspecific chromosome races in this respect. Müntzing (279) summarized 38 cases for which data on ecology and distribution were available and concluded that intraspecific chromosome races are probably almost always different ecologically.

The two races of *Phleum pratense* ($2x$ and $6x$), although occurring in the same locality, occupied ecologically different habitats (132). Diploid *P. alpinum* was obtained from Switzerland, the tetraploid forms from Scotland and northern Scandinavia (279). According to Gregor and Sansome (134), the tetraploid forms were decidedly more vigorous than the diploid. The distribution of *Dactylis aschersoniana* is limited, whereas the autotetraploid *D.*

glomerata has spread throughout the temperate zones of the world as an important forage grass (279). In *Festuca*, polyploid races had contributed to the extension and distribution of the genus (178, 180). As forage plants, tall fescue (*F. elatior* var. *arundinacea*, $2n = 42$) is known to be adapted to drier and more impoverished soils than meadow fescue (*F. elatior*, $2n = 14$).

To these examples may be added several others including *Glyceria fluitans* (75), *Poa annua* (250), *Agropyron junceum* (502, 503, 504, 356) and *A. elongatum* (503). Tetraploid ($2n = 20$) plants of *Anthoxanthum odoratum* have been reported several times but diploids only once (355), indicating a wider distribution of the tetraploid.

It is apparent that an imposing array of evidence has been accumulated indicating that polyploid species of the Gramineae tend to be adapted to a wider range of ecological conditions and to occupy wider geographical distributions than their diploid relatives. Exceptions have been noted, however. The case of *Puccinellia vahliana* (120) has been cited. Stebbins and Love (525) found that differences in drouth and heat tolerance in California grasses apparently were limited to allopolyploids; in their material intraspecific chromosome races (autopolyploids) did not show differences in distribution. Likewise, Nielsen (328) did not find evidence of regional segregation on the basis of chromosome number of races of *Panicum virgatum*. Bowden (39) compared the winterhardness of 100 species and varieties of angiosperm (none of them a grass, however) with their chromosome numbers and was unable to find a relationship between degree of polyploidy and winterhardness.

Origin of Polyploids in Experiments

In the forage grasses, polyploids have been obtained experimentally from three sources: (a) induction by colchicine or heat treatment, (b) twin seedlings, and (c) sporadic occurrence in genetic and breeding stocks.

Heat treatment was shown (378) to be effective in causing chromosome doubling in early mitoses of the young embryo of maize. This method has been applied successfully also to some other species but has not been used extensively in the forage grasses. Peto (368), in attempts to produce amphidiploid hybrids of *Triticum vulgare* \times *Agropyron glaucum*, applied a variety of heat treatments

to wheat florets following pollination with *A. glaucum*. Only one plant from over 13,000 treated florets showed chromosome doubling.

Discovery of the efficacy of colchicine in inducing chromosome doubling (34, 35, 322, 323) provided a generally and easily applicable method for production of polyploids. By use of colchicine, autotetraploids have been produced in *Lolium perenne* and *L. multiflorum* (147, 289, 495), *Sorghum vulgare* var. *sudanese* (cf. 289, 485), *Panicum miliaceum* (16), *Stipa lepidota* (523), *Dactylis aschersoniana* and *Festuca elatior* var. *pratensis* (312). A 12-ploid ($2n = 84$) plant of *Phleum pratense* was also produced (284). Chromosome doubling was induced in *L. perenne*, *F. elatior*, *D. aschersoniana* and *S. lepidota* by treatment of germinating seeds. Most of the plants were mixoploid, and, in *L. perenne*, $2x$ and $4x$ clones were established by repeated vegetative propagation with single tiller isolates (147). Colchicine treatment has been used also in production of fertile amphidiploids from sterile hybrids of *Agropyron glaucum* with varieties of *Triticum vulgare*, *T. durum*, *T. turgidum*, *T. dicoccum* and *T. pyramidale* (19, 370).

Polyembryony (586), resulting in twin seedlings, occurs relatively frequently in some grasses, *Poa pratensis* for example, and occasionally in other species. In most of the twins, the two seedlings are similar morphologically and have the same chromosome number; in some cases the twin plants differ phenotypically but have the same chromosome number; in other pairs the twin plants differ both phenotypically and in chromosome number. Ordinarily the deviating plants have one-half as many as or more than the normal number (termed haploids and triploids for convenience, although where a polyploid species is involved these terms are not strictly correct). Müntzing (280, 282) examined twin seedlings from species of 11 genera, including nine forage grasses. The frequency of plants with deviating chromosome numbers varied from 0 to 9% in the different species. Müntzing (282) concluded that the plant with the deviating number was almost invariably smaller and weaker as a seedling than the normal twin. On the other hand, Skovsted (508) found in some instances differences in chromosome number that were not associated with differences in size of seedlings. Also, he reported a higher frequency of twins with deviating number than Müntzing (282) found. Triploid and haploid plants of *P. pratense* and a haploid plant of *D. glomerata*

have been obtained from twin seedlings (346). It is evident that twin seedlings provide, in the grasses, a less fertile source of polyploids than colchicine treatment.

Ordinarily polyploids do not occur in sufficient frequency in untreated material to be of significance experimentally. Nevertheless, a few have been obtained, including autotriploid plants of *Pennisetum typhoides* (228) and *Phleum nodosum* (349) and auto-hexaploid plants of *Alopecurus pratensis* (188) and *Dactylis glomerata* (312). Occasionally, functioning of unreduced gametes has resulted in polyploids in crosses between species—a triploid F_1 from *Lolium loliaceum* \times *L. rigidum* (184) and a triploid ($2n = 66$) from *Saccharum spontaneum* ($2n = 56$) \times *Sorghum durra* (161). In *Paspalum urvillei* ($2n = 40$) \times *P. malacophyllum* ($2n = 40$) the F_1 had 40 chromosomes. In $F_1 \times P. dilatatum$ ($2n = 40$) the plants had 60 chromosomes (61). In the hybrid *Festuca arundinacea* ($2n = 42$) \times *F. pratensis* ($2n = 14$) the F_1 had 28 chromosomes. Progeny were obtained only from seed produced with open-pollination. Among these were plants with 35, 42, \pm 49, 63 and 77 chromosomes (342). Likewise, a 35-chromosome plant was obtained (335) from a seed produced by open-pollination on the F_1 of hexaploid *Festuca rubra* \times *Lolium perenne*.

Amphidiploids have been discovered in *Phleum pratense* ($2n = 14$) \times *P. alpinum* ($2n = 28$) (132, 134, 350), in *Festuca arundinacea* ($2n = 42$) \times *F. gigantea* ($2n = 42$)—in the fertile derived polyploid $2n = 84$ —(339), and in hybrids between species of *Triticum* and *Agropyron* (208–211).

Characteristics of Experimental Polyploids

Most determinations of effects of chromosomal reduplication are complicated by gene differences that may accentuate or obscure differences between the diploids and the induced polyploids. This difficulty may be obviated by use of genetically homozygous material or by vegetative propagation from plants that are sectorial chimeras or mixoploids of diploid and polyploid tissue. The latter method was used in *Lolium perenne* (147). Morphologically the autotetraploids were distinguishable from the related diploids in having somewhat wider, thicker and longer leaves, thicker but fewer tillers, longer leaf sheaths, and larger florets, spikelets, pollen grains and seeds. The stomata also were slightly larger, but the two types

could not be distinguished on that basis. Under favorable cultural conditions the $2x$ and $4x$ clones could, in most cases, be identified readily by appearance. There was, however, evidence of a differential response to chromosome doubling; in some pairs the morphological differences were slight (304). These effects of chromosome doubling on morphology are similar to those reported generally (36, 279, 521). Autotetraploids of *Stipa lepida*, likewise, had larger floral parts but differed in showing no evidence of broader and thicker leaves (523). In *Panicum miliaceum*, autotetraploids had larger spikelets and seeds (16).

A striking effect of chromosome doubling in *Lolium perenne* was the greater sensitivity to winter injury of the autotetraploids (304). This behavior was unexpected in view of the generally more northerly distribution of polyploids in the grasses, but is consistent with results obtained with tetraploid rape and tomatoes (482, 493). Also contrary to the usual effect of chromosome doubling, no differences were found between related $2x$ and $4x$ clones of *L. perenne* in maturity nor in growth rate, except in rapidity of production of new tillers (304). On the other hand, the tetraploids of *Stipa lepida* were all slower in growth and later in maturity than their diploid relatives (523).

In greenhouse experiments, relative yields were determined in eight pairs of diploid and tetraploid clones of *Lolium perenne* grown in soil and in gravel and subjected to different frequencies of defoliation by clipping (513, 514). Statistical analysis of the data showed an effect upon plant yield of chromosome doubling and that the effect was significantly altered by the genotype involved (pairs of clones from different original seeds), the medium in which the plants were grown, and the clipping treatment used for evaluation. In yield of tops the tetraploids did not differ significantly from the diploids when all clones and treatments were averaged. On the other hand, average yield of stubble and of roots of the diploids significantly exceeded that of the tetraploids. Variation in response to chromosome doubling was reported also in *Stipa lepida* (523). Some autotetraploids were more vigorous and others less vigorous than their related diploids. Triploids ($2n = 63$) of *Phleum pratense* and their seed progenies ($2n = 56$ to 64) were more vigorous and higher yielding than normal 42-chromosome plants (284, 286, 287). Of these, plants with $2n = 56$ (exactly $8x$) were most vigor-

ous of all. A 12-ploid plant ($2n = 84$) produced by colchicine proved to be inferior; its chromosome number was above the optimum for the genus (287).

In general, the effects of chromosomal reduplication in *Lolium perenne* on chemical composition were of small magnitude (535, 536). The tetraploids were higher in moisture, sucrose, total sugar and percentage of dry matter soluble in 80% alcohol. No difference was found in nitrogen content. The genotypes responded in a differential manner to chromosomal reduplication in regard to cellulose and lignin content. There have been reports of differences in chemical composition resulting from chromosome doubling in several other species, none of them forage grasses, however (cf. 26 for recent literature).

Autopolyploids obtained in experiments have been reduced in fertility compared with related diploid species, and autopolyploids in the forage grasses are not exceptional in that regard. Reduced fertility has been found in *Lolium perenne* and *L. multiflorum* (495), *Panicum miliaceum* (16) and *Stipa lepidota* (523).

Evolutionary Significance of Polyploidy

The importance of polyploidy in the formation of new species and in extending the range of ecological adaptation and geographical distribution of genera and species is now generally recognized (101, 107, 279, 521, *et al.*). Stebbins (521) stated that of the many processes which have been active in plant evolution, only polyploidy is well enough understood from the cytological point of view to permit a safe estimate of its rôle in species formation.

With reference to the Gramineae, Avdulov (21) concluded that polyploidy played little or no part in the evolution of major groups, even genera. Its effect has been confined to evolution within the genus and frequently within a section of the genus. Even within *Festuca*, evolution from the Bovinae to the Ovinae has occurred without change in chromosome number (242). The present viewpoint of the functions of polyploidy has been adequately summarized by Stebbins (521) whose views are in agreement with those expressed by Avdulov (21) and several other investigators. Stebbins (521) attributed to polyploidy a major rôle in the formation of new species and races of plants. These new forms owe their characteristics, in part, to effects of chromosome doubling, but more par-

ticularly to the bringing together and recombination of genes from diverse forms. Stebbins (521) distinguished between the origin of species and evolution itself, and concluded that polyploids have not been and could not be expected to be the starting point of a new evolutionary line. Compared with diploids, a polyploid complex tends to be a closed system. Huskins (156) expressed agreement with this hypothesis.

The dominant rôle of polyploidy in speciation usually has been attributed to allopolyploids (107, *et al.*), but Müntzing (279), although recognizing the importance of allopolyploidy, presented considerable evidence of the importance of autopolyploidy. Stebbins (521) concluded that the relative importance of chromosome doubling alone compared with allopolyploidy varies with different genera. Thus allopolyploidy is the important factor in *Crepis* (24, 521), whereas in *Tradescantia* autopolyploidy has assumed the dominant rôle (13).

Abundant evidence of the importance of polyploidy is found in the Gramineae. Included among the polyploids are most of the widely distributed and economically important forage grasses (294). In fact, relatively few of the important forage species are diploid—*Lolium perenne*, *Festuca elatior* var. *pratensis*, *Agropyron cristatum* (the Fairway strain but not the commercial forage strains which are autotetraploid), *Pennisetum glaucum* and *Setaria italica*.

MONOHAPLOIDS AND POLYHAPLOIDS

Haploids were divided by Katayama (*cf.* 159) into monohaploids (from diploid species) and polyhaploids (from polyploid species). Monohaploid (polyhaploid ?) plants of *Sorghum vulgare* occur relatively frequently and are characterized by being smaller than normal and highly sterile (51). At midprophase, synapsis of the chromosomes was nearly complete. Bivalents were observed at metaphase I, and dicentric bridges occurred at anaphase I.

Two polyploid plants of *Dactylis glomerata* were less than half the size of normal tetraploids and showed some resemblance to *D. aschersoniana*, the putative parent of *D. glomerata* (285). In one plant the anthers degenerated prior to meiosis. In the other, seven bivalents were formed regularly in meiosis, but no functional pollen was produced (285).

Polyhaploid plants (somatic number = 21) of *Phleum pratense*

were smaller and weaker than the hexaploids and were kept alive only with difficulty (349). The heads and spikelets especially were smaller, and the plants were both male and female sterile. Microsporogenesis was very irregular; the most frequent pairing in meiosis was seven bivalents plus seven univalents (349), but variations from seven bivalents plus seven univalents to 21 univalents occurred (237). The most striking irregularity was cell fusion in meiotic prophase, from two to 30 pollen mother cells fusing to form one large syncytium. At metaphase I, these large cells formed a single bipolar spindle with all bivalents on one regular equatorial plate (237).

Polyhaploids of *Poa pratensis* have been obtained several times (329, 533) from twin seedlings. One polyhaploid (329) was markedly different in size and vigor from its normal twin. The plant had 28 chromosomes plus a fragment, and produced a high frequency of aposporous embryo sacs, but the seed was not viable. A polyhaploid of *Stipa cernua* was shorter than normal but relatively vigorous, and was completely sterile. At meiosis, 35₁ occurred in about one-third of the sporocytes, with one or two bivalents plus univalents in the remainder (256).

ANEUPLOIDS

Variable and aneuploid chromosome numbers have been reported commonly in some of the species of *Poa*, particularly *P. pratensis* and *P. alpina*. In these species, the aneuploid numbers are perpetuated by apomixis. This subject will be considered in more detail later. Variable numbers have been found also in *Alopecurus alpinus* (120, 188) and *A. antarcticus* (188). Flovik (120) concluded that there was no constant number for *A. alpinus*, the variation being conditioned by autopolyploidy and by viability of aneuploids due to the very high chromosome number ($2n = 112, 114, 119$ to 122). A similar situation probably obtains also in *A. antarcticus* ($2n = 112$ to 116).

Aneuploid plants have been found occurring naturally or among the progenies of euploid parents in *Lolium perenne* (304), *Agropyron cristatum* (288, 308, 365), *Arrhenatherum elatius* (308), *Festuca arundinacea* (366), *Phleum pratense* (304) and *Dactylis glomerata* (275, 281, 308). Of these species, only *L. perenne* is diploid. The remaining species except *F. arundinacea* have meiotic

behavior characteristic of autopolyploids, and, hence, aneuploidy is expected.

The most extensive studies of the occurrence of aneuploids have dealt with *Dactylis glomerata*. Müntzing (275, 281) found 10% of aneuploids on the basis of "accurate values" and 19% on the basis of "total values" among the plants of eight progenies, all the parents of which were probably euploid. Among plants from open-pollinated populations, Myers and Hill (308) reported about 40% aneuploids, while in progenies of euploid plants, aneuploids occurred in frequencies varying from 5% to 27% (312). The incidence of aneuploidy is particularly high among the progenies of aneuploids. Among 76 plants of the progeny of a 29-chromosome plant, 29 had 28 chromosomes, two had 27, 30 had 29, 14 had 30, and one had 31 (275). Among the offspring of 27-chromosome plants, 26-chromosome plants were expected but none was obtained. Part of the plants were $2n=27$ and part $2n=28$. In all cases investigated there was a tendency for progenies of aneuploid plants to revert to the normal number of 28. In types with less than 28 chromosomes, gametic and zygotic selection are important factors, while in plants with more than 28, the extra chromosomes are lost frequently during meiosis (281).

From backcrosses of *Dactylis aschersoniana* \times *D. glomerata* to *D. aschersoniana*, Müntzing (281) obtained plants with chromosome numbers from 14 to 20. In backcrosses of the F_1 to *D. glomerata*, the chromosome numbers of the progeny varied from 22 to 41 and one plant had 56 chromosomes. Thus there was established an almost continuous series of numbers from 14 to 41. Selfed progenies of pentaploid plants ($2n=35$) likewise contained plants with somatic numbers varying from 28 to 41.

Among 119 plants from seed produced by open-pollination (pollen from diploid plants) on an autotriploid plant of *Lolium perenne*, 25% had $2n=14$, and the remainder had 15 to 18 chromosomes (300). In progenies of various trisomic plants pollinated with pollen from diploids, the incidence of trisomics varied from almost 50% to less than 5% (304).

An aneuploid series was established in *Phleum* (349) from triploid *P. nodosum* ($2n=21$) \times *P. pratense* ($2n=42$) and in the progeny of 63-chromosome plants of *P. pratense* obtained from twin seedlings (287). In the former case the chromosome numbers varied from 33 to 44 and in the latter from 56 to 64.

In the euploid and aneuploid plants of *Dactylis*, Müntzing (281) found a progressive increase in vigor through the series 14, 21, 28 and 35. Plants with chromosome numbers between these euploids showed reduced vigor, the reduction being greatest in each case approximately at the mid-point, *i.e.*, the greatest deviation from a multiple of 7. The 56-chromosome plant was weak and died before flowering. In *Phleum* the entire group of plants with chromosome numbers from 56 to 64 were more vigorous than normal hexaploids, while plants with 56 to 58 chromosomes were more vigorous than those with 59 to 61 (287).

It is well known from the investigations of *Datura* that different trisomics frequently may be recognized from the phenotypes of the plants. Similar but less complete results have been obtained for the pentasomics of *Dactylis glomerata* (281) and the trisomics of *Lolium perenne* (304). In the latter case, however, one of the trisomics had no observable effect upon the phenotype, at least when the plants were grown in the greenhouse.

MEIOSIS

Normal Meiosis

Meiosis has been observed in more than 80 species of grass (exclusive of *Triticum*, *Aegilops*, *Secale*, *Avena*, *Oryza* and *Zea*). In most reports only general statements were made; details of the extent of observations and the frequency of various irregularities have been given only in relatively few cases. Furthermore, the investigations frequently were limited to one or a few plants of the species. Thus more complete studies of larger numbers of plants may reveal in many species features of meiotic behavior not now known.

Meiosis has been reported to be regular in *Phalaris coerulescens*, *P. paradoxa* (359), *P. canariensis* (202, 359), *Zizania aquatica* (376), *Bromus mollis* (219), *B. carinatus* and *B. catharticus* (526), *B. arizonicus* (527), *Puccinellia angustata* (120), *Andropogon furcatus*, *A. scoparius*, *A. virginicus*, *A. glomeratus*, *Festuca capillata*, *F. elatior* var. *pratensis*, *Spartina patens* var. *junceae* and *S. cynosuroides* (71, 72, 74), *Hordeum* spp. (14), *Eleusine indica* (224), *Agropyron tenerum* (= *A. trachycaulum*), *A. richardsonii*, *A. caninum* and *A. dasystachyum* (365), *Paspalum gayanus* and *Poa resinulosa* (490), and *Oryzopsis hymenoides* and *Stipa viridula* (185).

Irregularities of Meiosis

Quadrivalents and other multivalents occur with such regularity in certain instances that the species are considered to be autopolyploids. The meiotic behavior of such species will be discussed later (cf. Meiosis in Autopolyploids). Low frequencies of quadrivalents, resulting presumably from autosyndesis, have been reported in several other species (cf. Types of Polyploids).

Aside from multivalent formation, the most commonly reported irregularity of meiosis has been the occurrence of univalents at metaphase I and lagging chromosomes at anaphase I. Unpaired chromosomes at metaphase I have been reported in *Calamagrostis neglecta*, *Puccinellia phryganodes*, *Trisetum spicatum* (120), *Agrostis nebulosa* (546) and *Dactylis aschersoniana* (202). In these cases the behavior of the univalents was not reported. The occurrence and behavior of univalents at metaphase I were investigated in *Lolium perenne* (291). The univalents usually became oriented on the equatorial plate somewhat later than the bivalents and divided equationally at anaphase I. The daughter half chromosomes were sometimes left in the cytoplasm at telophase I, but, more commonly, they were included in the daughter nuclei and lagged and were lost in the second division. This type of univalent behavior in meiosis occurs frequently and had been reported among forage grasses in *Festuca elatior* (312), *Dactylis aschersoniana* (281), in the interspecific hybrids *Phleum pratense* ($2n = 42$) \times *P. subulatum* (292), *P. pratense* ($2n = 14$) \times tetraploid *P. alpinum* (349), *Agropyron junceum* \times *A. repens* (352), *Dactylis aschersoniana* \times *D. glomerata* (281), and *Festuca elatior* ($2n = 42$) \times *Lolium perenne* (312). (cf. also Meiosis in Autopolyploids.) In contrast, the univalents of polyhaploid *P. pratense* ($2n = 21$) passed at random to the poles and split only rarely, while in triploid *P. nodosum* ($2n = 21$) an average of 4.68 univalents was observed at metaphase I and only 1.81 univalents were seen to divide at anaphase I (349). Univalents at metaphase I and laggards at anaphase I have been reported also in *Bromus inermis* (217), *B. villosus* (27), *Deschampsia alpina* (120), *Agropyron junceum*, *A. repens* (352), *Poa pratensis* (18), *P. caesia*, *Alopecurus fulvus* and *A. myosuroides* (84). The quantitative relationship of metaphase I univalents and anaphase I laggards was not reported in these cases. Church (71, 72) reported univalents and laggards in several spe-

cies. In the species reported in one paper, however, he (71) made an effort to select for study plants that produced poor pollen. Therefore, the meiotic behavior may not be typical for normal plants of the species.

Lagging chromosomes at anaphase I were observed in *Pennisetum typhoideum* (381), *Bromus marginatus* and *B. rubens* (27). Presumably these laggards resulted from unpaired chromosomes at metaphase I. Relatively high frequencies of laggards at anaphase I were found in *Agropyron amurense*, *A. intermedium*, *A. repens* and *A. trichophorum* (9). Irregularities of meiosis (not described) and considerable sterility occur in *Paspalum intermedium* (490). A case of complete asynapsis that was heritable was found in *Alopecurus myosuroides*, and some plants of *A. pratensis* were partially asynaptic (188). In addition to plants of *Lolium perenne* with low frequencies of metaphase I univalents, a type of asynapsis, conditioned by a single gene, has been studied in which two or rarely four univalents occur in from 20% to over 80% of the sporocytes (304).

Two cases of failure of the spindle mechanism to function properly have been reported in forage grasses. In a derivative of *Lolium perenne* \times *Festuca elatior*, Darlington and Thomas (103) reported lack of spindle compactness and failure of the spindle to converge on the poles. Krishnaswamy (224) described a type of spindle abnormality in *Eleusine coracana* in which the bivalents did not orient regularly at metaphase nor disjoin in an orderly manner at anaphase I. The non-orientation of bivalents in *L. perenne* (291) may be a manifestation of the same phenomenon.

Non-homologous pairing was first reported in midprophase of maize (258). In the forage grasses this phenomenon has been observed in a triploid F_1 plant of *Lolium loliaceum* \times *L. rigidum* (184) and in autotriploid *L. perenne* (300). Likewise, in haploid *Sorghum vulgare* there was nearly complete synapsis at midprophase that was attributed largely to non-homologous pairing (51).

The random distribution of chromosomes of bivalents to the two poles at anaphase I is one of the cardinal principles in cytogenetics. This assumption of random distribution has been extended also to the third set of chromosomes of triploids (101) and the extra chromosomes of aneuploids. When this hypothesis was tested in triploid *Datura*, however, an excess of sporocytes with a majority

of extra chromosomes passing to one pole and a deficiency of the more nearly equal distributions was found (487, 488). On the other hand, in plants of *Lolium perenne* with two extra chromosomes ($2x + 1 + 1$), the observed ratio of 7-9 and 8-8 distributions at anaphase I did not deviate significantly from the expected 1:1 (300). Likewise, in the autotriploid (301) the distribution of extra chromosomes gave a satisfactory fit to the binomial $(a + b)^7$, expected on the hypothesis of randomness.

Meiosis in Autopolyploids

Studies of meiotic behavior in autopolyploids have dealt most commonly with frequency of multivalents at diakinesis and metaphase I. In *Dactylis glomerata* the number of quadrivalents varied from zero to seven among sporocytes with an average of 3.5 to 3.8 for the species (275, 281, 307, 308). Similar results were obtained in *Arrhenatherum elatius* (307, 308), tetraploid *Agropyron cristatum* (307, 308), *Hordeum bulbosum* (31) and autotetraploid *Lolium perenne* (303). Kattermann (203) reported four to seven quadrivalents per sporocyte in *Arrhenatherum elatius*. Variations among plants in average quadrivalent frequency have been reported in *Arrhenatherum elatius*, *Dactylis glomerata*, *Agropyron cristatum*, *Phleum pratense* and autotetraploid *Lolium perenne* (307-311, 297, 302, 303). In *D. glomerata* the extremes among plants were 2.62 and 4.91 (311). Multivalent formation in *Anthoxanthum odoratum* varies widely in different sporocytes. Trivalents, quadrivalents, sexivalents, octavalents and rings of 10 and 12 chromosomes were observed (203), the associations varying from 10 bivalents to $1_{XII} + 1_{IV} + 2_{II}$ in 50 sporocytes. Similar behavior was reported by others (355, 359).

The occurrence and behavior of unpaired chromosomes at metaphase I were investigated in tetraploid *Agropyron cristatum*, *Arrhenatherum elatius* (307, 308), *Phleum pratense* (302), *Dactylis glomerata* (297, 307-311), and autotetraploid and autotriploid *Lolium perenne* (300, 303). In each species the percentage of sporocytes with one or more univalents varied among plants. The greatest variation was among 84 first inbred generation plants of *D. glomerata*; the percentages of metaphase I sporocytes with univalents ranged from 0.8% to 97% (311).

The orientation of the univalents was variable in each species.

Some were oriented on the equatorial plate along with the bivalents and multivalents prior to the initiation of anaphase I; others were scattered through the sporocyte during metaphase I but became oriented some time before the completion of anaphase I. The univalents, upon orientation, divided equationally, and the daughter half chromosomes reached the poles in a majority of cases in time to be included in the interphase nuclei. Some, however, were left in the cytoplasm where they were seen at interphase as chromatin clumps. The daughter half chromosomes behaved abnormally in the second division. Some were scattered in the cytoplasm near the poles and probably were included in the microspore nuclei at telophase II. Others were oriented on the plate at metaphase II, lagged at anaphase II and occurred as chromatin clumps or micro-nuclei in the quartets.

The interrelationships of chiasma frequency, quadrivalent frequency, percentage of metaphase I sporocytes with univalents, percentage of anaphase I with laggards, and percentage of quartets with micronuclei and chromatin clumps have been investigated by correlation and covariance analysis in *D. glomerata* and autotetraploid *L. perenne*. Chiasma frequency was positively correlated with quadrivalent frequency but negatively correlated with percentage of metaphase I univalents in both species. Metaphase I univalent frequency was not correlated with quadrivalent frequency in either species when unrelated plants were studied (297, 303, 309, 310). By the analysis of covariance it was shown that variations in chiasma frequency did not account for all differences in quadrivalent frequency and incidence of metaphase I univalents (297). Among inbred plants of *D. glomerata* (within inbred families), however, there was a significant negative correlation between quadrivalent frequency and incidence of metaphase I univalents (311). The nature of the interrelation of chiasma, quadrivalent, and metaphase I univalent frequency has been discussed (297, 311).

X As expected from behavior of the metaphase I univalents, their frequency was positively correlated with frequency of anaphase I laggards and of micronuclei in the quartets. Likewise, there was a positive correlation between the latter two characters. In *D. glomerata*, quadrivalent frequency was not correlated with frequency of laggards at anaphase I, indicating that quadrivalents were not a source of anaphase I laggards (297, 309-311). Furthermore,

the analysis of covariance showed that variations in metaphase I univalents were sufficient to account for all significant differences in anaphase I laggards (297). In *Lolium perenne*, however, frequency of anaphase I laggards was positively correlated with frequency of quadrivalents, indicating that some of the laggards in this species may have resulted from improper disjunction of the quadrivalents (303).

The irregularities of meiosis and, consequently, the low fertility of autopolyploids have been attributed to multivalent association of chromosomes during synapsis and the tendency of multivalents to disjoin unequally at anaphase I (101, 221, 222, *et al.*). Consequently, there has been a general tendency to accept multivalent frequency as a criterion of the relative meiotic irregularity of autopolyploids. On the other hand, Müntzing (279) attributed much of the infertility of autopolyploids to physiological disturbances and upsets in genic balance accompanying chromosomal reduplication. Randolph (379) recognized that meiotic irregularities cause some sterility but agreed that physiological and genic disturbances are the important causes. In *Antirrhinum* (515) and maize (119), differences were found in fertility of autotetraploids of different origin that were not associated with variations in quadrivalent frequency. Similar results were obtained in inbred progenies of *Dactylis glomerata*, but in this species quadrivalent frequency is not a reliable criterion of regularity (297, 311). Only 2.5% of the anaphase I sporocytes had 13-15 distribution, while the remainder were normal, indicating that regular disjunction of the quadrivalents was the rule. Furthermore, quadrivalents did not contribute significantly to lagging at anaphase I in *D. glomerata* (297, 311).

Unequal disjunction of quadrivalents occurs commonly in *Lolium perenne*, however. Only 52% of the anaphase I sporocytes had a 14-14 distribution, while 40% had 13-15 and 8% had 12-16 (303). Similar results have been obtained in several other autopolyploids (see 303 for literature). Quadrivalent frequency also contributed in *L. perenne* to the incidence of laggards at anaphase I.

The importance of unpaired chromosomes at metaphase I, with their subsequent loss or random inclusion in daughter nuclei, as a source of aneuploid gametes has been stressed (297, 311). In *D. glomerata*, frequency of metaphase I univalents was negatively correlated with fertility among plants of inbred progenies (311).

Metaphase I univalents also occurred commonly in autotetraploid *L. perenne* and probably were an important factor in causing aneuploidy and reduced fertility in that species (303).

There are at least three major types of meiotic irregularity in autopolyploids, namely: (a) unequal disjunction of members of the multivalents, (b) incomplete disjunction of the multivalents, resulting in lagging and dividing univalents at anaphase I, and (c) unpaired chromosomes at metaphase I. Of these, the first type has been recognized most commonly. The feature of meiotic irregularity of greatest importance varies with the species, and multivalent frequency alone will not always be a reliable criterion of fertility and stability of the autopolyploid (297, 303).

Kostoff (221, 222) concluded that in species with short chromosomes autopolyploids would tend to be more regular in meiosis because of lower chiasma frequency and hence fewer quadrivalents. This hypothesis was adopted to account for fertility of autotetraploid sea plantains allied to *Plantago maritima* (109). In *Dactylis glomerata* and *Lolium perenne*, however, chiasma frequency was negatively correlated with incidence of metaphase I univalents. Hence, decrease in chiasma frequency, although it would tend to reduce the number of quadrivalents, would, in these species, result in greater irregularity of meiosis because of the greater frequency of univalents at metaphase I (297).

The relation of meiotic behavior in autotetraploids to behavior in the related diploid was studied in *Lolium perenne*, using pairs of diploid and tetraploid clones isolated from single seedlings (147). In some pairs the chiasma frequency per chromosome of diploid and autotetraploid was not different, but in others the tetraploid had a lower frequency than the related diploid (303). In this regard Upcott (566) postulated that chiasma frequency per chromosome might be lower in polyploids than in comparable diploids due to a delay of pairing resulting from the larger nucleus of the polyploid. From the literature on this problem (303) it is evident that the relationship varies with different species and even among clones of the same species, as in *L. perenne*.

In *L. perenne* there was no significant correlation between diploid and autotetraploid in chiasma frequency, percentage of metaphase I with univalents, percentage of anaphase I with laggards, and percentage of quartets with micronuclei. There was evidence in one

clone of an upset in timing balance in meiosis resulting from chromosome doubling. The meiotic regularity of an autotetraploid in *L. perenne* could not be predicted from the behavior of the diploid from which it was produced (303).

Statistically significant differences among clones in regularity of meiosis must generally result from heritable differences. The progeny test provides, however, the only critical proof of such heritable differences. From progeny tests in *Dactylis glomerata* (311), heritable variations among plants were found for chiasma frequency, average number of quadrivalents per sporocyte, percentage of metaphase I with univalents, percentage of anaphase I with laggards and percentage of quartets with micronuclei. The existence of heritable variations in these characters indicates the possibility of selecting for greater regularity of meiosis in autopolyploids.

Comparative studies of meiotic behavior of parental clones and their first inbred generation progenies of *Dactylis glomerata* have been reported (311). Among eight comparisons, the average quadrivalent frequency of the inbred did not differ significantly from that of the parent in six cases but was significantly higher in two cases. The most striking effect of inbreeding was on incidence of univalents at metaphase I. On the average, inbred progenies had two to three times as many unpaired chromosomes at metaphase I as their respective parents. Similar increases were found in frequencies of laggards at anaphase I and micronuclei in the quartets. The greater incidence of asynapsis in the inbreds could not be attributed to decreased chiasma frequency. The behavior of second inbred generation progenies (299) was similar throughout to that in the first generation.

It has been suggested that some species which behave cytologically as allopolyploids have developed from autopolyploids by a process of chromosomal differentiation (101, 279). According to Darlington (101), gene rearrangements rather than intragenic changes would be the principal factor in such differentiation. There is a dearth of experimental evidence on the effectiveness of various amounts and kinds of rearrangements in inhibiting random pairing among the four or more homologues of the autopolyploid. Skirm (507) reported an autotetraploid form of *Tradescantia* with predominantly bivalent pairing attributed to structural heterozygosity and to doubling after fertilization. Because of their interference

with regularity of synapsis, inversions might be expected to play an important part in limiting synapsis to particular pairs in an autopolyploid. From comparisons of bridge and fragment frequencies at anaphase I in pairs of diploid and autotetraploid clones of *Lolium perenne*, it was evident, however, that single inversions of the size dealt with had no very appreciable effect on random pairing between the four homologues (303).

CHROMOSOMAL REARRANGEMENTS

Associations of four or more chromosomes at diakinesis and metaphase I have been reported in several diploid species, including *Briza media* (204 to 206), *Puccinellia vahliana* (120), *Agrostis nebulosa* (546), *Dactylis aschersoniana* (281) and *Festuca pratensis* (377). These have been attributed by the authors (except Flovik (120)) to structural hybridity. The most analyzed case is *Briza media*. From studies of the progenies of structurally heterozygous plants and of intercrosses between ring-forming and non-ring-forming plants it was established clearly that the ring of four chromosomes resulted from an interchange (205, 206). In a cross of two non-ring-forming plants the progeny plants had a ring of four chromosomes (206). Some of the quadrivalents reported in polyploid species (see Types of Polyploids) may also have resulted from reciprocal interchanges rather than homology between chromosomes of different genomes. *Anthoxanthum odoratum* behaves cytologically like an autotetraploid (203, 355, 359). Associations of more than four chromosomes occur frequently, and this has been attributed by the authors to structural hybridity. A more nearly complete cytogenetic analysis of this species seems warranted. Associations of more than three chromosomes were observed occasionally in autotriploid *Pennisetum typhoides* and were attributed to segmental interchanges (228). Reciprocal interchanges, resulting in semisterility, were produced in *Pennisetum typhoides* by treatment of resting seeds with X-rays (229).

The presence of dicentric chromatid bridges and acentric fragments at anaphase I and, less frequently, at anaphase II has been shown to result from crossing over in heterozygous inversions (258, 260). Bridges and fragments have been observed in *Phalaris brachystachys* (359), *Bromus carinatus* (526), *Agropyron junceum* and *A. repens* (352), autotriploid *Pennisetum typhoides* (228),

Lolium perenne (291, 303), *Dactylis glomerata* (297, 308 to 311), *Phleum pratense* (302), *Festuca elatior*, $2n = 14$ and $2n = 42$ (312), *Anthoxanthum odoratum* (355), *Agropyron amurense*, *A. intermedium*, *A. repens* and *A. trichophorum* (9). One plant of *Pennisetum typhoides* obtained from X-ray-treated seed was heterozygous for an inversion (229).

INTERSPECIFIC AND INTERGENERIC HYBRIDIZATION

Natural and Controlled Hybrids

Interspecific and intergeneric hybrids and their cytogenetic characteristics have been reported commonly in the Gramineae. Investigations of this nature involving the cereals, particularly *Triticum* and the related genera *Aegilops*, *Secale* and *Haynaldia*, have been reviewed (1), and studies of hybrids of maize and maize relatives have been summarized (262). In the forage grasses, over 200 interspecific and intergeneric hybrids have been reported. Of these, 93 were naturally occurring, 119 artificial or controlled, and 17 both natural and controlled. Identification of natural hybrids and their parents is, at best, circumstantial and must be based upon proximity of the hybrids to the putative parents in nature, sterility of the hybrids, chromosome numbers and meiotic behavior, and comparative characters of the hybrids and the putative parents. Since not all of the data are available in many instances, it is probable that some of the reports of natural hybrids may be erroneous. Ullmann (565) tabulated most of the natural and controlled hybrids reported prior to 1936. Extensive lists of natural hybrids have been reported in the flora of Denmark (11) and New Zealand (81, 82). Cugnac (90) has discussed the high frequency of hybridization among grass species in nature.

Bromus. Most of the studies of interspecific hybrids in *Bromus* (especially in 65, 66, 88, 89, 92, 93, 95-98, 100), were for the purpose of elucidating problems of phylogeny and systematics in the genus. Cugnac (88) reported that between the species *B. sterilis* L. and *B. madritensis* L. there exists a series of intermediates formed by *B. rigidus* Roth., *B. gussonei* Parl and *B. macrantherus* Hack. The F_1 plants of *B. sterilis* \times *B. madritensis* resembled *B. madritensis*, were irregular in meiosis and produced little pollen (89). In *B. sterilis* \times *B. macrantherus* the F_1 resembled *B. sterilis* and meiosis was irregular. Apart from its sterility the F_1 of this

cross resembled *B. gussonei* in every respect (89). Later, chromosome number determinations supported the assumption of the hybrid origin of *B. gussonei* (100). In hybrids involving *B. grossus*, *B. secalinus* and *B. arduennensis* the F_1 was completely fertile in each case. Cugnac (96) concluded that these three species should be considered varieties of one collective species. Hybrids of members of this collective species with the closely related *B. arvensis* and *B. macrostachys* were sterile.

B. arduennensis and *B. grossus* differ in pubescence of spikelets and in several other characters. The *B. grossus* characters were completely dominant in F_1 and a dihybrid segregation occurred in F_2 . Pubescence was conditioned by a single factor, and all other differential characters segregated *en bloc* as if determined by a single gene (93, 95).

The characters of *B. arduennensis* were completely recessive in crosses with *B. arvensis*, *B. macrostachys*, *B. secalinus*, *B. grossus* and *B. grossus nitidus*. In contrast, the characters of *B. macrostachys* were completely dominant in crosses with *B. arduennensis*, *B. grossus nitidus*, *B. arvensis* and *B. squarrosus*. In *B. grossus* \times *B. arvensis* the F_1 was sterile and resembled *B. grossus* morphologically (97).

Knowles (220) attempted to cross *Bromus mollis* with 13 species representing five sections of *Bromus*. Hybrids were produced with *B. racemosus*, *B. arenarius*, *B. rubens*, *B. madritensis* and *B. carinatus*. The chromosome pairing in F_1 paralleled the morphological similarities of the parents. In the hybrid with the morphologically similar *B. racemosus*, bivalent pairing was nearly complete. On the other hand, in the F_1 with *B. carinatus*, which is distinct morphologically and geographically from *B. mollis*, bivalent formation was rare.

The F_1 plants of *Bromus hordeaceus* \times *B. mollis* (334, 341) were intermediate between the parents in morphology and fertility. The fertility of the hybrids indicates a close relationship between these species.

Stebbins and Tobgy (526) reported $21_{II} + 7_I$ in the F_1 of *B. carinatus* ($2n = 56$) \times *B. catharticus* ($2n = 42$). The univalents appeared to be the chromosomes of seven long bivalents observed in *B. carinatus*. The F_1 plants were completely sterile despite relatively regular meiosis. The authors suggested that *B. carinatus*

was an amphipolyploid derived from a hybrid of *B. catharticus* with some diploid species of the *Bromopsis* section. In *B. arizonicus* \times *B. carinatus*, F_1 plants showed a maximum pairing of seven medium sized trivalents, 14 medium sized bivalents, 14 medium sized univalents, and seven large univalents, the latter coming from *B. carinatus* (527). In this hybrid extensive inversion hybridity was indicated by the high frequency of bridge-fragment configurations.

Festuca and Lolium. Interspecific and intergeneric hybrids of *Festuca* and *Lolium* have been found in nature (11, 58, 82, 335, 339, 483, 539, 589, also cf. 565) and have been produced by controlled cross pollination (88, 146, 168, 175, 178, 179, 183, 184, 218, 312, 332, 333, 342, 553, 591, 592). Meiotic behavior in F_1 of *Festuca elatior* var. *arundinacea* \times *F. gigantea* has been investigated (339, 366). Bivalents and univalents, with occasional quadrivalents and trivalents were observed at metaphase I. Peto (366) reported an average of 13.9 univalents (range from 10 to 19) per sporocyte, while Nilsson (339) found a maximum of 14 univalents. The F_1 was pollen sterile and rarely produced seeds with open-pollination. One such seed produced a plant with 84 chromosomes, an amphipolyploid (338).

Among the six species of *Lolium* recognized by Jenkin and Thomas (183), 11 interspecific hybrids have been investigated (178, 179, 183, 184). In general, seven bivalents were formed in meiosis in each hybrid, although as many as 40% of the sporocytes in certain crosses had two, or rarely four or more, univalents at metaphase I. Dicentric bridges and acentric fragments at anaphase I in six of the hybrids might be interpreted to indicate chromosomal differentiation among species, although such inversion bridges occur commonly in plants of *L. perenne* (291, 303). The anthers dehisced and 20% to 25% good pollen was produced in *L. perenne* \times *L. italicum* (= *L. multiflorum*), *L. perenne* \times *L. rigidum* and *L. rigidum* \times *L. loliaceum* (triploid plant). In the remaining crosses the anthers did not dehisce (179, 183).

Hybrids of *Lolium* spp. \times *Festuca* spp. (178) are particularly interesting, since these genera have been placed in different tribes on morphological characters. In *L. perenne* \times *F. elatior* var. *pratensis* the 14 chromosomes of the F_1 occurred regularly as seven bivalents at metaphase I, with a chiasma frequency only slightly

lower than in the parents (366). Despite the regularity of meiosis, the F_1 plants were completely male sterile (the anthers did not dehisce) but produced some seed from backcrosses to the parents. Sterility of the hybrids was attributed to genic causes.

In F_1 *L. perenne* \times *F. elatior* var. *arundinacea* an average of 32.4% of the 28 chromosomes occurred as univalents, 55.2% as bivalents, 4.3% as trivalents, 5.7% as quadrivalents, and 2.4% as quinquevalents (366). In another study the number of univalents varied from zero to three, the number of bivalents from five to 12, and the number of quadrivalents from zero to four (312). The results indicate considerable homology between *L. perenne* and *F. elatior* chromosomes, and between chromosomes of the genomes of *F. elatior*. Consistent with these results, one to five quadrivalents were observed at diakinesis and metaphase I in *F. elatior* var. *arundinacea* (312). Similarly in F_1 of *L. perenne* \times *F. rubra* the frequency of bivalents varied in different sporocytes from seven to 12, indicating pairing between *Lolium* and *Festuca* chromosomes and between chromosomes of different *Festuca* genomes (335).

Poa. *P. arachnifera* \times *P. pratensis* was one of the first controlled hybrids recorded among the forage grasses. According to Vinall and Hein (582), the cross was made by Oliver in 1908, and was repeated later by Brown. The primary objective was to combine the heat and drouth tolerance of *P. arachnifera* (Texas bluegrass) with good forage quality of *P. pratensis*. Some selections from this cross have appeared promising in preliminary trials, but no new commercial variety has yet been developed from the material.

The hybrids of *P. pratensis* \times *P. alpina* (4, 7, 283) and *P. compressa* \times *P. pratensis* (48, 49) are of interest primarily for their contribution to analysis of the problem of apomixis in *Poa* (see Apomixis). In the latter cross, an unreduced gamete of *P. compressa* was fertilized by an approximately reduced gamete of *P. pratensis*. The F_1 plant was intermediate between the parents in several characters, but in other respects, particularly retention of green leaves during midsummer, it appeared to be superior to either parent. Furthermore, the hybrid was rather highly fertile (49). Despite the fact that both parents were highly apomictic, the hybrid was completely or nearly completely sexual; the F_2 showed extreme segregation, plants occurring with various combinations of the characters of the parents. In extensive F_2 and F_3 populations, not a

single plant appeared to be superior to either parent, a majority being distinctly inferior in vigor, leafiness and other important characters (304).

Controlled hybrids of *P. nemoralis* × *P. pratensis* (cf. 565) and *P. pratensis* × *P. glauca* (7) have been obtained, but their characteristics were not reported. In addition, several natural interspecific hybrids have been recorded (11, 82, 251, 565).

Dactylis. *Dactylis aschersoniana* × *D. glomerata* has been recorded several times in nature (11, 152, 281, 538, 565) and has been produced by controlled hybridization (146). Meiosis in the F_1 is characteristic of an autotriploid, trivalents, bivalents and univalents occurring at metaphase I (281). The F_1 plants were male sterile but produced seed in backcrosses with either parent. According to Ullmann (565), Vogt produced the intergeneric hybrid *Cynosurus cristatus* × *D. glomerata*.

Melica. In F_1 of *Melica imperfecta* × *M. torreyana* pairing is normally as bivalent; univalents occur only rarely, yet the plants are nearly 95% seed sterile (189). Allotetraploids, from chromosome doubling in the F_1 were vigorous, high in pollen fertility, variable but exceeding the hybrid in seed fertility, and formed two to five multivalents at diakinesis. Behavior of the F_1 is similar to that reported in *Lolium* × *Festuca* hybrids (see page 357). Increased fertility and meiosis in the allotetraploids resembles the condition in *Primula kewensis* (565). Many of the naturally occurring polyploid grasses, in which multivalents occur at meiosis, may prove to have arisen in a similar manner.

Agropyron, *Elymus*, *Triticum*, *Aegilops*, *Secale*. Several interspecific hybrids in *Agropyron* have been reported (11, 146, 352, 353, 501, 502, 565, 575). Only one of them, *A. junceum* × *A. repens* (352), has been studied in detail. In four hybrid plants the average pairing at metaphase I varied from $13_{II} + 9_I$ to $9_{II} + 17_I$, and, in addition, a few trivalents were observed. The hybrids were male sterile and failed to produce seed with open pollination.

The intergeneric hybrids of *Triticum* × *Agropyron* have been investigated more extensively than any others among the forage grasses. Excellent reviews of the literature, particularly pertaining to crossing relationships, have been published recently (509, 510, 564, 588). Much of the work with these hybrids has been done in Russia where the primary objective has been production

of a perennial wheat. The possibility of producing new forage plants was recognized, however (209–211, 233, 560). Likewise, the work in Canada, particularly in Saskatchewan, has been directed towards the production of a large-seeded, perennial forage plant, combining some of the winterhardiness, disease resistance and perenniality of *Agropyron* with the seed size and better forage quality of *Triticum* (588).

Among numerous species of *Agropyron*, *A. elongatum* and *A. glaucum* have been used most extensively and successfully in crosses with *Triticum* (17, 19, 20, 37, 186, 187, 209, 233, 324, 367–370, 372, 486, 506, 561–564, 567–579). Successful crosses have been reported also with *A. intermedium*, *A. trichophorum* and *A. junceum* (20, 208–211, 324, 355, 509, 510, 562, 563, 572, 574–579). Smith (509, 510) obtained one hybrid plant of *T. aestivum* × *A. cristatum* that died before flowering, and several hybrid seeds of *T. durum* × *A. amurense* and *T. aestivum* × *A. amurense*. The "*A. amurense*" may have been mislabeled and actually have been a strain closely allied to *A. intermedium* (510).

Crosses using several other species of *Agropyron* have been attempted without success. These include *A. caninum*, *A. tenerum* (*A. trachycaulum*), *A. sibiricum*, *A. loliodes*, *A. repens*, *A. turczaninovii*, *A. desertorum*, *A. prostratum*, *A. orientale*, *A. subsecundum*, *A. smithii*, *A. inerme*, *A. dasystachyum*, *A. ciliare*, *A. semicostatum* and *A. spicatum* (509, 510, 562, 575, 588).

For the *Triticum* parent, varieties of *T. aestivum* and *T. durum* have been used most extensively in hybrids with *Agropyron*. Verushkin (575) reported, however, that some *Agropyron* species will cross with forms in all three sections of *Triticum*. White (588) succeeded in crosses with *A. elongatum* and *A. glaucum* using *T. dicoccoides*, *T. dicoccum*, *T. durum*, *T. turgidum*, *T. polonicum*, *T. timopheevi*, *T. pyramidale* and *T. vulgare* (*T. aestivum*) as female parents. Östergren (354) reported the hybrid *T. turgidum* × *A. junceum*, and Popowa (372) reported *T. timopheevi* × *A. elongatum*.

Greater success has been attained in crosses using *Triticum* as the female parent (560, 579, 588), and the seed set was better on the average with *T. durum* × *Agropyron* spp. than with *T. aestivum* × *Agropyron* spp. (17, 510, 588). Furthermore, the crossing results varied with different varieties of *Triticum* and different strains of *Agropyron* (186, 211, 510, 560, 575, 579, 588).

Investigations of meiotic behavior in *Triticum* × *Agropyron* hybrids were reported by Vakar (567–572). In F_1 of *T. vulgare* × *A. elongatum* the maximum association of chromosomes varied in different hybrids— 28_{II} , $21_{II} + 14_I$ and $14_{II} + 28_I$. On the basis of the pairing behavior, Vakar (568, 570) concluded that the genomes of *A. elongatum* were Aa, Ba, Da, X_1 and X_2 where Aa, Ba, and Da are *Agropyron* genomes homologous with the A, B, and D genomes of *T. vulgare*. One of these genomes was less homologous than the other two and failed to pair in some hybrids ($14_{II} + 28_I$). In hybrids with 28_{II} , autosynopsis between X_1 and X_2 genomes was assumed. These conclusions were supported also by pairing observed in *T. durum* × *A. elongatum* (568); in these hybrids maximum associations of 14_{II} or occasionally 21_{II} occurred. Sapegin (486) observed $21_{II} + 7_I$ as the maximum association in F_1 of *T. vulgare* × *A. elongatum*. Popowa (372) found a maximum pairing of $21_{II} + 7_I$ in F_1 of *T. timopheevi* × *A. elongatum* which he attributed to pairing of A and G of *T. timopheevi* with Aa and Ba of *A. elongatum* and autosynopsis of X_1 with X_2 . In *A. elongatum* an average of three quadrivalents with low frequencies also of quinquevalents, sexivalents and octavalents occurs (367). The occurrence of quadrivalents is consistent with Vakar's (569) postulation of homology between the X_1 and X_2 genomes. In the F_1 of *T. vulgare* × *A. elongatum*, Peto (367) reported very complex pairing including all of the types of association observed in *A. elongatum* but in different frequencies.

In F_1 *T. vulgare* × *A. glaucum*, Vakar (568) reported variations from 6_{II} to 14_{II} with an average of 10_{II} , while in *T. durum* × *A. glaucum* a maximum of 7_{II} was observed. He (568) concluded that *A. glaucum* had the genomes Aa, Da and X_2 . Peto (367) found an average of 6.2_{II} in *T. dicoccum* (Vernal emmer) × *A. glaucum* and 5.5_{II} in *T. durum* × *A. glaucum*. On the other hand, Sapegin (486) observed only two or three, rarely four, bivalents in *T. vulgare* × *A. glaucum*. According to Sipkov (506), pairing varied in different hybrids of *T. durum melanopsis* × *A. glaucum* from 2_{II} or 3_{II} to $14_{II} + 7_I$.

The behavior in crosses involving *A. intermedium* has been reported (208–211). In *T. durum* × *A. intermedium* ($2n = 28$) the frequency of bivalents varied from zero to two. In *T. durum* × *A. intermedium* ($2n = 42$) the F_1 plants produced unreduced (35 chro-

mosomes) gametes. In backcrosses to wheat, sesquidiploids were produced, while upon selfing of the F_1 , amphidiploids resulted. In some crosses, 95 to 98% of the selfed progeny of the F_1 were amphidiploids. Some of the amphidiploids were reported to be exceedingly valuable forage plants (210, 211).

The fertility, particularly self-fertility (male fertility ?), of the F_1 plants varied widely among crosses of different species and even among different hybrids involving the same species. Verushkin (575) reported that (a) in *T. vulgare* \times *A. elongatum* there was a tendency for fertility, (b) in *T. durum* \times *A. intermedium* (cf. 211) and *T. durum* \times *A. trichophorum* individual plants were self-fertile, (c) in *T. vulgare* \times *A. intermedium* and *T. vulgare* \times *A. trichophorum* the F_1 very rarely set seed following selfing, and (d) in *T. durum* \times *A. elongatum* the F_1 was entirely self-sterile. Similar results were reported by others (20, 187, 561). In *T. vulgare* \times *A. elongatum* the most fertile F_1 hybrids were obtained when varieties of soft wheat were used and the self-fertile forms were those with 21_{II} to 28_{II} whereas those with 14_{II} were self-sterile (568). On the other hand, there was no relation between fertility and meiotic behavior in hybrids involving *A. glaucum* (561, 569). In cases in which the F_1 has been self-sterile, it usually has been possible to obtain progenies by backcrossing to the parents.

In the F_1 the *Agropyron* characters tended to be dominant and in some cases heterosis was observed (561, 562, 575, 588). In F_2 and later generations from the self-fertile hybrids there was extreme segregation (most characters of both parents were recovered according to White, 588), an increase in fertility and self-fertility, and a decrease in percentage of perennial plants. Similar results were obtained in progenies of backcrosses of the F_1 to *Triticum*, but in these progenies there was a tendency to revert rapidly to the *Triticum* type, and, particularly, a rapid reduction of the percentage of perennial plants. The progenies of self-fertile hybrids tended to become stabilized with characteristics intermediate between the parents and with a chromosome number of 56 (568, 572). For satisfactory forage, types more wheat-like than any plants in the F_2 progenies were required (187). In general, the hybrids of *A. glaucum* were more suitable for forage than those of *A. elongatum* (186, 588).

Favorskii (118) reported successful crosses of *A. intermedium*

with three species of *Aegilops*. These hybrids are of interest primarily because of the relation of *Aegilops* to *Triticum* (1).

Using *Secale cereale* as the female parent, successful crosses have been made with *A. intermedium* (575), *A. cristatum* (223), *A. sibiricum*, *A. repens* and *A. trichophorum* (509). At meiosis in the F_1 of *Secale cereale* \times *A. cristatum* ($2n = 28$), bivalents were almost always formed, a maximum of seven occurring (117). This was interpreted by the author to indicate pairing of *Secale* and *Agropyron* chromosomes. Since *A. cristatum* ($2n = 28$) behaves cytologically like an autotetraploid (308), Favorskii's (117) interpretation probably is questionable. Smith (509) used six other species of *Agropyron* in attempted crosses with *S. cereale* and none was successful.

Species of *Elymus* have been used successfully in intergeneric hybrids in three instances. Cugnac (91) and Cugnac and Belval (99) reported hybrids of *Elymus riparius* \times *Agropyron caninum*, and TSitsin (561) obtained crosses of *Secale cereale* \times *Elymus junceus*. Hertzsch (146) reported hybrids of *Melica ciliata* \times *Elymus arenarius*. Poddubnaja-Arnoldi (371) attempted a number of *Triticum* \times *Elymus* crosses and found neither embryo nor endosperm development. In *T. timopheevi* \times *E. araliensis* and *T. vulgare* \times *E. dahuricus*, however, the embryo and endosperm occasionally passed through early stages of development before aborting. Smith (509) pollinated *T. aestivum*, *Secale cereale* and *Hordeum vulgare* with several species of *Elymus* but none of the crosses was successful.

Danthonia. Seven natural interspecific hybrids of *Danthonia* were reported from the flora of New Zealand (82).

Calamagrostis. Six natural interspecific hybrids (11, 67) and two intergeneric hybrids—*Anmophila arenaria* \times *Calamagrostis epigeios* (11) and *Calamagrostis tenella* \times *Agrostis alba* (cf. 565)—have been recorded.

Agrostis. Numerous natural interspecific hybrids have been reported (11, 82, 122; cf. 565). The probability of extensive natural hybridization of *Agrostis tenuis*, *A. alba* and, possibly, *A. palustris* in New England is indicated by the work of Stuckey and Banfield (605). Single panicles, judged on morphological ground to be *A. tenuis*, were collected from old pastures and meadows, and spaced planted progeny plants were grown from the seed of each

panicle. In part of the progenies, morphological variations were within the limits expected of *A. tenuis*, but in others the plants showed varying combinations of vegetative and panicle characters of *A. tenuis*, *A. alba*, and, occasionally, *A. palustris*. Furthermore, the chromosome numbers were variable within progenies. Rarely plants were found with 28 or 42 chromosomes, but usually the numbers were intermediate. There was no evident relation between chromosome number and morphology. Further investigations of this interesting problem are in progress.

The possibility of an intergeneric hybrid involving *Agrostis* has been suggested. Sokolovskaya (511) postulated that *A. verticillata* may form a connecting link between *Agrostis* and *Polypogon* and that *P. litoralis* is possibly a hybrid of *P. monspeliensis* and an *Agrostis* species.

Alopecurus. Ten natural interspecific hybrids have been reported (11; cf. 565). In a review in Plant Breeding Abstracts, Johnsson (188) is credited with describing interspecific hybrids in *Alopecurus*. Only *A. antarcticus* \times *A. pratensis* is mentioned specifically. Since this paper apparently is not available in libraries in this country, the additional hybrids could not be checked.

Phleum. Meiotic behavior in interspecific hybrids in *Phleum* have been discussed previously (see Types of Polyploids). The hybrids seem to offer little of practical value, but have been of particular importance in the analysis of the chromosomal relationships in hexaploid *P. pratense*.

Stipa. Six interspecific hybrids involving eight species of *Stipa* have been produced experimentally (256a). The hybrids include *S. lepida* \times *S. pulchra*, *S. pulchra* \times *S. cernua*, *S. cernua* \times *S. lepida*, *S. cernua* \times *S. leucotricha*, *S. comata* \times *S. neomexicana*, and *S. californica* \times *S. occidentalis*. All hybrids were completely sterile, but fertility was induced in *S. cernua* \times *S. pulchra* by use of colchicine. In *S. lepida* \times *S. pulchra* there were 14 to 18 bivalents, zero to two trivalents, and no or one quadrivalent at meiosis, while in *S. pulchra* \times *S. cernua* there were 10 to 19 bivalents, no or one trivalent and no or one quadrivalent. No conclusions can yet be drawn regarding the chromosomal homologies in this genus.

Spartina. On the basis of chromosome numbers and the occurrence of the two species in the locality in which *S. townsendii* originated, it was assumed that *S. townsendii* arose by chromosome

doubling in the hybrid of *S. alterniflora* × *S. stricta* (154, 155). This explanation has been accepted generally. It was questioned, however, by Chevalier (69) who suggested that *S. townsendii* has affinities to *S. glabra pilosa* and *S. merrillii* (*S. glabra* × *S. polystachya*) which deserve consideration. *S. townsendii* is an outstanding example in the Gramineae of a species that has arisen, supposedly by polyploidy following interspecific hybridization, in comparatively recent times—it was discovered in 1870. Since that time it has spread widely, occupying the area formerly occupied by the putative parents and exceeding greatly the parents in distribution. Moreover, it is an important forage grass along coastal areas of northwestern Europe (56, 57, 154).

Phalaris. The F_1 plants of *P. arundinacea* × *P. tuberosa* were male sterile but fairly female fertile (2 to 4% of the florets set seed) in the presence of the parental species. In meiosis, $12_{II} + 4_I$ were regularly formed. In F_2 (open-pollinated seed, probably from *P. tuberosa* pollen) there was a great diversity of types, some different from either parent (182). Vogt (cf. 565) reported *P. arundinaceae* × *P. canariensis* but the hybrid was not described.

Paspalum. The hybrids of *P. urvillei* × *P. malacophyllum* were male sterile but produced seed when pollinated with pollen from *P. urvillei* or *P. dilatatum*. From these backcrosses plants of F_1 × *P. urvillei* and F_1 × *P. dilatatum* were obtained, in addition to plants identical with the F_1 . Only unreduced gametes functioned in the F_1 plants and the unreduced eggs were capable of parthenogenetic development (61). *P. urvillei* and *P. malacophyllum* both produce uniform progeny, suggesting parthenogenesis, but the F_1 plants had $2n = 40$, indicating that reduced gametes functioned in production of the hybrids.

Setaria. In the F_1 of *S. italica* × *S. viridis* there were nine bivalents at metaphase I and otherwise regular meiosis (243). The plant produced 70% sterile pollen and 50% of the spikelets were empty. Plants of the F_2 segregated from very low fertility to as high as the parents. Li, Pao, and Li (246) found $9_{II} + 9_I$ usually in meiosis in F_1 *S. faberii* × *S. italica* and *S. faberii* × an F_2 plant of *S. italica* × *S. viridis*.

Sorghum. Hybrids of *S. vulgare* × *S. halepense* have been reported (30, 198, 270). Univalents, bivalents and quadrivalents were found at meiosis in the F_1 (198). The F_1 plants were fertile

and extreme segregation occurred in F_2 , resulting in a complete range of intermediate types. Over one-fourth of the F_2 plants survived the winter. The F_3 plants were more fertile than F_2 and retained their hybrid vigor (30). Mikharlovskii (270) also found segregation in F_2 and reported that in most F_2 plants $2n = 30$ but in some $2n = 40$. *S. dimidiatum* and *S. purpureo-sericeum* hybridize easily. *S. dimidiatum* is characterized by the lower half of the lower glumes being coriaceous, the upper half papery. In hybrids with *S. purpureo-sericeum*, this character behaves as a simple recessive (443).

Saccharum and related genera. *Saccharum officinarum* and *S. spontaneum* have been crossed successfully with *Sorghum vulgare* (38, 165, 166, 167, 273, 505, 543) and *S. halepense* (165). In hybrids of sugar cane variety P.O.J. 2725 ($2n = 107$) with *Sorghum vulgare* (273) three types of F_1 plants were obtained: (a) normal or sugar cane type with $2n = 118$, (b) intermediate type with $2n = 64$, and (c) dwarf type with $2n = 64$, all plants of which died before flowering. One or both of these species of *Saccharum* have been crossed successfully also with *Narenga narenga*, *Erianthus sara*, *E. arundinaceus*, *E. ravennae*, *Zea mays*, *Imperata cylindrica* and *Bambusa arundinacea* (165, 166, 480). A majority of the hybrids were pollen-sterile; some failed to flower, but others were fertile (165).

Other hybrids. Additional hybrids that have been recorded are as follows:

- Puccinellia distans* \times *P. maritima* (11)
- P. distans* \times *P. retroflexa* (11)
- P. maritima* \times *P. retroflexa* (11)
- Arundo conspicua* \times *A. fulvida* (11)
- Hordeum sativum* \times *H. bulbosum* (230)
- H. jubatum* \times *H. vulgare* (375)
- H. nodosum* \times *H. vulgare* (375)
- H. jubatum* \times *Secale cereale* (46, 47, 85)
- Arrhenatherum elatius* \times *Avena pubescens* (cf. 565)
- Stipa viridula* \times *Oryzopsis hymenoides* (185)
- Phippsia algida* \times *P. concinna* (120)
- Anthoxanthum aristatum* \times *A. odoratum* (355)
- Brachypodium pinnatum* \times *B. silvaticum* (11, 65)
- Deschampsia chapmani* \times *D. tenella* (82)

Deyeuxia avenoides × *D. quadriseta* (82)

D. billardieri × *D. foisteri* (82)

D. foisteri × *D. pilosa* (82)

Dichelachne crinita × *D. sciurea* (82)

Hierochloe fraseri × *H. redolens* (82)

Microlaena avenacea × *M. stipoides* (82)

Trisetum antarcticum × *T. youngii* (82)

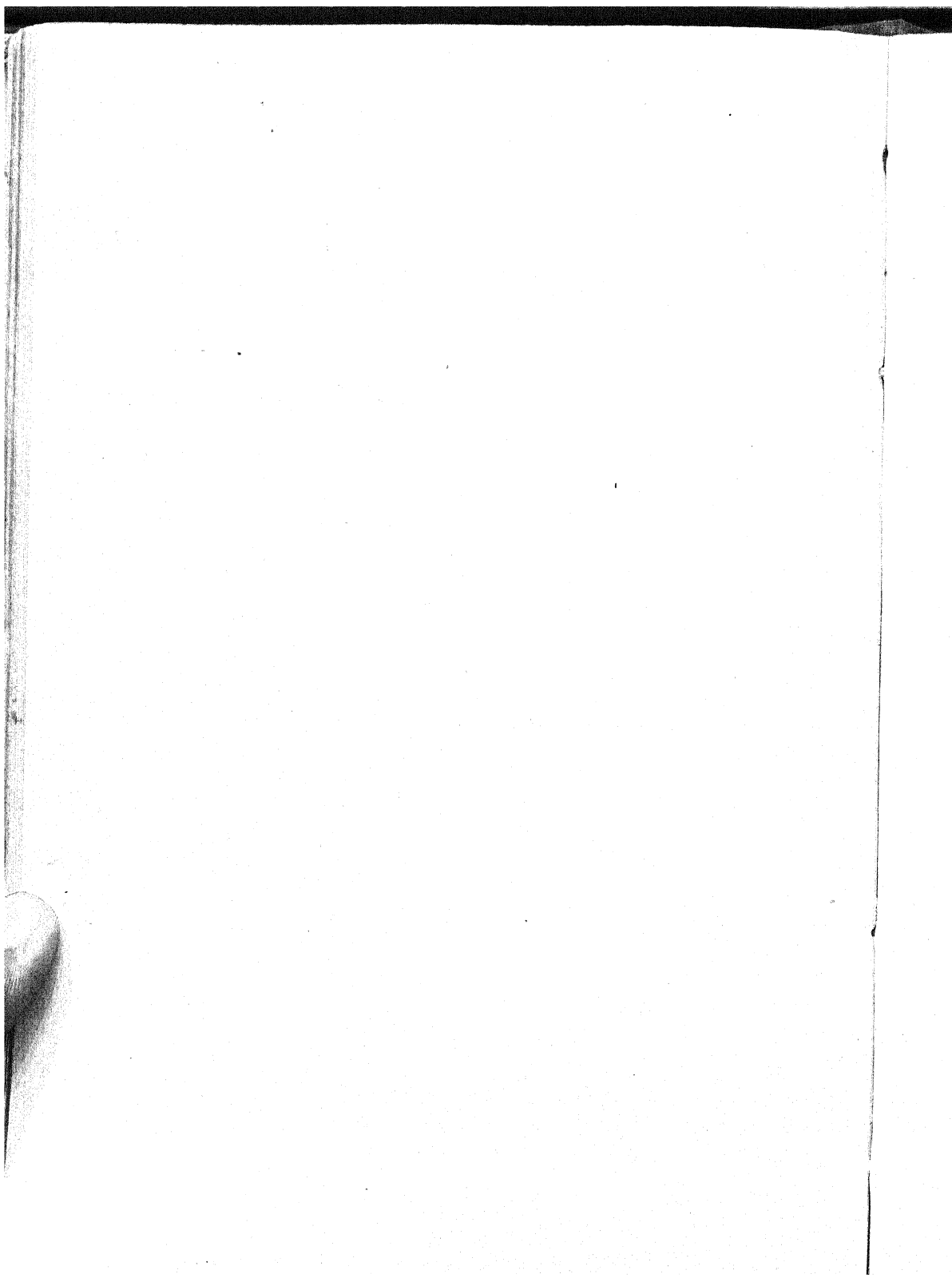
Glyceria fluitans × *G. plicata* (565)

G. fluitans × *L. perenne* (565)

Variations in Seed Set in Reciprocal Crosses

Variations in cross-compatibility in reciprocal interspecific and intergeneric hybridizations have been encountered commonly. Ordinarily, the cross has been most successful when the parent with higher chromosome number was used as the female. Explanations for this difference have been proposed (276, 544, 545, 585). In the forage grasses, exceptions to this general behavior have been encountered. In interspecific and intergeneric hybrids of *Lolium* and *Festuca* the cross most commonly succeeded best when the female parent had the lower chromosome number (178). Likewise, *Triticum* × *Agropyron* crosses were more successful when *Triticum* was the female parent (588, *et al.*).

(To be concluded in next issue)



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CYTOLOGY AND GENETICS OF FORAGE GRASSES

(Concluded)

W. M. MYERS

INHERITANCE OF QUALITATIVE CHARACTERS

Sorghum

The genetics of *Sorghum vulgare* has been investigated more completely than that of any other grass except *Zea mays* and *Hordeum vulgare*, and a large number of genes are known. In 1938 Stephens and Quinby (531) stated that approximately 50 factor pairs had been determined by them in the previous 12 years. Many of these genes were listed in summary tables (265). Ranga-swami Ayyangar and his associates in India have published numerous reports of inheritance in *Sorghum* (383 to 385, 405 to 413, 420 to 453, 457 to 469). Many characters were the same as those studied by investigators in the United States (265, 531), but others represented new factors. In addition to the numerous genes reported from natural mutations, Quinby and Karper (374) obtained 72 abnormalities from X-ray treatment among approximately 2,000 progenies. A majority were seedling characters but nine were mature plant characters.

Many of the known genes were discovered and their behavior studied in the grain sorghums. Since all of the sorghums, including forage and grain types, Sudan grass and broom corn, so far as is known, can be intercrossed freely and produce fertile hybrids (531), the inheritance may be considered applicable to forage sorghums and Sudan grass. There are recent reviews of the genetics of *Sorghum* (e.g., 265, 385, 386, 531, 532).

Several genes have been reported conditioning chlorophyll abnormalities of seedlings and adult plants (194, 199, 265, 374, 434, 446, 457), and at least 18 different factors affect the presence, intensity and distribution of color in various plant parts including

coleoptile, leaf sheaths and blades, stems, midribs, spikelets, spikelet hairs, glumes and anthers (83, 129, 148, 265, 374, 383, 405 to 407, 411, 412, 429, 430, 433, 440, 445, 448, 451, 460, 461, 465, 466, 471, 531, 532, 593). Seed color is determined by a series of factors conditioning the shade, intensity and distribution of color in the pericarp, nucellar layer and aleurone (383, 407, 408, 425, 428, 467, 469, 497, 498, 531, 581). Monogenic inheritance has been found for other seed characters (196, 423, 447, 453, 468), dwarfing (195, 265, 374, 410, 413, 449, 499) and numerous modifications of various plant parts (83, 374, 382, 384, 423, 424, 426, 434, 435, 438, 439, 441, 454, 459). Several genes condition characters of the panicle, spikelets and glumes (200, 201, 265, 422, 427, 431, 434, 436, 439, 442, 447, 581), expression of awns (463, 464, 498, 499, 531, 581) and male sterility (201, 421, 432, 529). Three factors condition resistance to loose and covered smuts (264, 537), and a single gene causes resistance to *Pythium* root rot (40, 110).

Cases of pleiotropy include a gene conditioning absence of ligules and of pulvini in the inflorescence (434), a gene for weak midrib that inhibits the expression of long awn (434), barbing of the awns and feathery stigma (409, 458, 462), and length of awns and of stigma and style (420). Method of pollination is changed by a single gene which, in the presence of a gene for papery glumes, conditions cleistogamy (437). Contrary to the usual condition, Karper (195) reported a dominant tall mutation of frequent occurrence that increased plant height about 40%. Also a case of maternal inheritance has been reported (197).

The linkage relationships of a considerable number of the known genes in sorghum have been investigated. Martin (265) listed 59 cases of independent inheritance and stated that at least five of the ten linkage groups in sorghum are required to account for these results. Rangaswami Ayyangar and his associates (304, 411, 423, 426, 428, 433, 434, 435, 458, 459) also have reported a number of cases of independence. Linkage has been found in the following instances:

1. The Q B Gs group: Stephens and Quinby (531) reported 3-point linkage tests in which the crossover percentages were Q (13.2) B (11.3) Gs. Q determines blackish *vs.* reddish purple plant and black glumes in the presence of P. B is the factor for brown nucellus, and Gs conditions normal *vs.* green striped plants.

The association of Q and B in varieties had been noted previously (383). In addition, a yellow midrib factor (Y^{md}) is linked with Q with 35% crossing over, but the relation of Y^{md} to B and Gs was not determined (448).

2. The D Rs P group: In 3-point experiments (532) the cross-over percentages were D(10.9) Rs (16.4) P. D is the factor for dry *vs.* pithy stalks, Rs for red seedling stems, and P for purple plant color. The total crossover percentage of 27.3 between D and P agrees satisfactorily with 30% reported by Rangaswami Ayyangar, *et al.* (452). White seedlings (w_s) is linked with Rs (199), with 41.3% crossing over, and Martin (265) credited Stephens and Quinby with finding linkage between red coleoptile (apparently Rs) and midrib color (m). Whether this is the yellow midrib (Y^{md}) of Rangaswami Ayyangar and Sankara Ayyar (448) is not clear from the literature. If so, this may provide the connecting link between the Q B Gs and D Rs P groups.

3. Gs_2 Ms_2 A V_{10} group: Stephens and Quinby (604) established the gene order ms_2 -a- v_{10} for genes conditioning male sterility, awns and virescent seedlings. To this Stephens (530) added green-striped (Gs_2). The gene order is gs_2 (23%) ms_2 (11%) a (9%) v_{10} . Previously it was reported (459) that presence of awns (a) was linked with feathery stigma (18% crossing over), feathery stigma with hairy leaf tip (25% crossing over), and hairy leaf tip with presence of awns (43% crossing over). The position of hairy leaf tip and feathery stigma genes in relation to gs_2 and v_{10} has not been determined.

4. Pa, Z group: Panicle shape (loose *vs.* spindle, Pa_1pa_1) shows 1.0% crossing over with Zz for pearly *vs.* chalky grains (447).

5. Liguleless, axillary panicle shoots: These two genes show 0.01% crossing over (439).

6. Purple markings in the shoots and roots of seedlings: Rangaswami Ayyangar and Venkataramana Reddy (465) reported 18% crossing over between these characters.

7. Smut susceptibility with juicy stalk: Swanson and Parker (537) noted a relationship between smut susceptibility and juicy stalk but suggested that fundamentally the association may have been with sweetness rather than juiciness of the stalk.

8. Yellow seedlings and waxy endosperm: Karper (265) observed linkage (26.5% crossing over) between these characters.

9. Yellow leaves and stems and pericarp color: Stephens and Quinby (265) found linkage (13% crossing over) in this case.

Eleusine coracana

Among the genes investigated in this species are duplicate factors for albinism (389, 398), a factor for pericarp color (403) and seven genes determining presence, distribution, intensity and shade of plant color (388, 390, 397). One of the basic plant color factors interacts with two other factors to condition presence and intensity of grain color (400). Three genes condition glume length (387), three genes panicle shape (401, 402, 580), and two genes male sterility (404).

Pennisetum typhoideum (= *P. glaucum*)

Genes conditioning six types of chlorophyll deficiency (191, 392) dwarf plant (191), four types of sterility (191), sugary grains (360) and two panicle characters (394, 396) have been reported. Two complementary factors differentiate golden yellow from bluish green seeds (360).

Setaria italica

A case of albinism, conditioned by a single factor, has been reported (416). The presence, intensity and distribution of purple plant color is conditioned by four genes (243, 261, 419). Anther color is determined by an allelic series—brownish-orange dominant to yellow dominant to white (243, 393, 415). Four factors interact to produce seed-coat (lemma and palea) colors (243, 414), a dominant factor produces white instead of black pericarp (243), endosperm color may be conditioned by two factors (243, 244), and waxy endosperm is recessive to non-waxy (244). Plant height and seedling growth habit are conditioned by single genes (243, 395). Lax ear-head is distinguished from normal by a single factor (455), while "palmatic" ear-head is conditioned by two factor pairs.

Color of bristles (purple *vs.* green) is conditioned by three genes (243). The presence of bristles is determined by a basic factor, X, and the length of bristles is dependent upon two factors, L₁ and L₂, that are expressed in the presence of a third factor, E, but not in the presence of e (417). The absence of spikelets on the tips of the bristles is monogenic (418).

Three linkage groups were reported by Li, Li and Pao (243):

1. Ga W br group: In studies of anther color, a deficiency of brown (Br) was obtained and was accounted for by the linkage (38.7% crossing over) of Br in repulsion with a gametophyte gene, Ga. Gametophytes with ga were assumed to be unable to compete successfully with Ga. White pericarp, W, was also linked with br (12.3% crossing over). The authors postulated the gene order Ga W br.

2. In Pr₁ Pr₂ group: Abnormal ratios for bristle color were accounted for by assuming linkage of Pr₁ and Pr₂ with 20.9% crossing over; these genes were linked also with In, the plant color intensifier, the gene order being In (9.1%) Pr₁ (20.9%) Pr₂.

3. Seed-coat (lemma and palea) color gene, P and Pr₃ group: The basic factor for purple plant color, P, was linked with one of the bristle color genes, Pr₃, and these were linked with one of the seed-coat color genes, B, Vi or R, the gene order being X (19.4%) P (27.1%) Pr₃ where X is one of the seed-coat color genes.

In considering these linkage values, it should be borne in mind that they were obtained in segregating populations of *Setaria italica* × *S. viridis*. Although meiosis appeared normal, the elimination of gametes was evidenced by the reduced fertility of F₁ and F₂. Thus some of the distorted ratios, explained on the basis of linkage (243), may possibly have resulted from differential elimination of gametes.

Lolium

Chlorophyll-deficient seedling lethals, sterile dwarfs, lethal green seedlings and other defective types conditioned by single genes occur commonly in inbred progenies (169, 170, 173, 174, 304, 337). Complementary dominant factors condition the presence of color in the base of tillers (172). In hybrids of *L. perenne* × *L. multiflorum* a single dominant factor conditions a substance in the roots causing fluorescence (86, 248, 591, 592). The same, or a similar, gene was reported in Wimmera ryegrass (553).

Echinochloa

In *E. colona* var. *frumentacea* two factors conditioning anthocyanin pigmentation and one factor determining male sterility have been reported (456).

Alopecurus pratensis

Duplicate factors resulting in ratios of 15:1 and 3:1 of normal to albino seedlings, and several other chlorophyll-deficient types, both lethal and semi-lethal, have been observed in inbred progenies (344). In a later paper Nissen (345) reported complementary factors for chlorophyll development.

Panicum miliaceum

A single dominant factor conditioned anthocyanin pigmentation (190) and a second factor intensified the pigmentation (399). Three cumulative dominant factors conditioned degree of hairiness of the plants, and four factors interacted to produce various colors of seed (399).

Dactylis glomerata

Stapledon (519) found chlorophyll-deficient seedlings in 27% to 40% of the inbred progenies studied. In many cases there were fewer albino or virescent seedlings than expected and the deficiency was attributed to stray pollen. Also Nilsson (338) reported chlorophyll-deficient seedlings in inbred progenies, in some of which the segregation did not fit a monohybrid ratio. Later it was demonstrated in three families that the inheritance of albino and yellow seedlings was tetrasomic, a behavior explainable on the basis of the cytological behavior of the species (290, 293).

Phleum pratense

The genetical investigations related to chromosomal association in this species have been reviewed in the section "Origin of Polyploidy". In addition to the work (25, 78, 302, 306, 587) already cited the frequent occurrence of albino seedlings and other chlorophyll-deficient types in inbred progenies was noted (32, 141, 176, 338). Witte (590) found recessive segregates in one progeny that had degenerate pistils and ovules.

Festuca elatior L.

Albino seedlings, conditioned by a single recessive factor, were obtained in *F. elatior* (28, 192). The inheritance of a dwarf, compact form, conditioned by a single dominant factor, has been reported (479).

INHERITANCE OF QUANTITATIVE CHARACTERS

No cases have been reported in the forage grasses of detailed investigations on inheritance of quantitative characters. Heritable variations of a quantitative nature have been determined, however, for several characters including self and general fertility, height of plant, vigor, tillering, width and length of leaves, number of leaves, resistance to disease, winterhardiness, drouth resistance, earliness, carotene content, hydrocyanic acid content and mineral content. One or more of these characters have been investigated in *Sorghum vulgare* var. *sudanense* (83, 115, 123, 151), *Lolium perenne* (173), *Dactylis glomerata* (143, 295, 305, 338, 494, 519), *Bromus inermis* (143, 554), *Agropyron cristatum* (288), *Festuca elatior* (143, 338), *Festuca rubra* (177, 338, 341, 583), *Phleum pratense* (78, 142, 305, 338) and *Andropogon furcatus* (234).

VARIATIONS IN NATURAL POPULATIONS

No such extensive or detailed investigations of the genetics of natural populations as those carried out in *Drosophila* (107) have been reported in the forage grasses. Some information has been accumulated, nevertheless, on the importance of natural selection and on variations between and within natural populations of these plants. The generally superior adaptation of seed from local or domestic sources (171, 518, *et al.*) recognized by most agronomists, is a result of the operation of natural selection on the genetic constitution of the population. Turesson (555, 557-559) observed phenotypic and genetic differences among populations of *Festuca* and of *Dactylis glomerata* from different habitats. Kemp (207) reported the emergence in closely grazed permanent pastures of types of *Poa pratensis* and *Dactylis glomerata* adapted by growth habit to close grazing, and Jenkin (180, 181) observed in *Lolium perenne* a distinct tendency for mass divergence of type under different environmental conditions. Similar observations were reported in *Phleum pratense* (132, 540), *Dactylis glomerata* (133, 517, 520) and *Bromus inermis* (598).

The existence of morphologically and physiologically different geographical races is a result of the same process of divergence in natural populations under pressure of selection. Such geographical differentiation has been reported in *Bromus inermis* (325), *B. mollis* (219), *B. carinatus* (140, 603), *Panicum virgatum* (87, 476),

Andropogon furcatus (234, 476), *Agropyron smithii*, *Bouteloua gracilis* (476), *B. curtipendula* (351, 476) and *Deschampsia caespitosa* (80).

APOMIXIS

A comprehensive review of apomixis in the angiosperms has been published (522). According to the classification proposed by Fagerlind (114) and favored by Stebbins (522), the two main types of apomixis are vegetative apomixis and agamospermy, or apomixis through seed production. Included in the former category are the so-called viviparous forms of *Festuca ovina* (120, 556, 558, 559), *Poa alpina* (120, 283), *P. alpigena* (120), *P. arctica* (120), *Deschampsia alpina* (80) and *P. bulbosa*. As pointed out by Stebbins (522), no careful cytological analysis of this type of reproduction has been reported. The expression of vivipary (proliferation) appears to be influenced, at least in some forms, by environmental factors (80, 327). In *Deschampsia caespitosa* northern ecotypes are more or less viviparous when grown in California, although they are normally sexual in their natural habitats (80).

Among the forage grasses, agamospermy, in the form of agamogony, has been studied most extensively in the genus *Poa*, particularly in *P. pratensis* and *P. alpina*. Apomixis in these two species was postulated by Müntzing (277) who found that parent plants with aneuploid chromosome numbers produced morphologically uniform progenies with the same chromosome number. It should be noted, however, that Zollikofer (599) suggested earlier the possibility of apomictic seed formation in *P. alpina*. Müntzing (283) later published the results of more extensive investigations confirming the hypothesis of apomictic seed production and the usefulness of the progeny test for determining the extent of apomixis. The progeny test was used also by Åkerberg (3, 5, 6, 7) in *P. pratensis* and *P. alpina* and, subsequently, in these species by others (50, 52, 53, 336, 340, 548). These investigations have revealed a range in type of reproduction from forms that are almost completely apomictic to forms that are completely, or nearly completely, sexual. Considerable differences in frequency of the various types have been noted by the different authors. Tinney and Aamodt (548) found a great preponderance of apomictic types and of types in which only a few per cent of the progeny plants indicated sexual

origin. Brittingham (50), on the other hand, found a considerably higher incidence of sexual reproduction.

The cytological basis for apomixis in *P. pratensis* was discovered by Åkerberg (6, 7, 8) whose findings were later confirmed and extended (214, 547). Somatic apospory was found, the embryo-sac initial being a cell of the nucellus. Ordinarily, in the apomictic forms the products of meiosis degenerated and were replaced in function by the aposporous embryo sac. Embryo development ordinarily started prior to fertilization but endosperm development apparently was dependent upon fertilization. Thus, the necessity of pollination for apomictic seed development (3, 4, 8, 111, 340) probably results from the necessity of fertilization for endosperm formation. In a sexual biotype no evidence was found of development of an embryo sac from a nucellar cell (6).

The occurrence of "haploid" and "triploid" plants among the progeny of certain biotypes indicates the occasional parthenogenetic development of reduced eggs and fertilization of the aposporous egg (6, 50, 283). Åkerberg (7) and Müntzing (283) concluded that apospory and parthenogenesis (pseudogamy) were distinct processes, either one occurring occasionally without the other. Similar behavior has been found in other plants (522).

In contrast with *P. pratensis*, generative apospory occurs in *P. alpina* (139, 283). Meiosis was not observed in apomictic biotypes, the first division of the macrospore mother cell being mitotic (139). In this species, also, pseudogamy occurred; embryo development started without fertilization, but endosperm development was dependent upon fertilization of the polar nuclei (139). In sexual forms of *P. alpina* with somatic chromosome numbers varying from 22 to 31, meiosis occurred and macrospores were capable of producing an embryo sac (139). The cytological basis for apomixis in *P. palustris* was reported to be similar to that in *P. alpina* (212, 213). In *P. arctica* apospory occurs similar to the condition in *P. pratensis* (112).

Other *Poa* species, including *P. alpigena*, *P. glauca* (111, 120) and *P. compressa* (49), have been reported on the basis of the progeny test to reproduce by apomixis. Apomixis was found also in *Calamagrostis obtusa* and possibly in *C. purpurea* (528), and has been suggested in *Arctagrostis latifolia* (120).

Paspalum dilatatum, *P. urvillei* and *P. malacophyllum* plants pro-

duce uniform progenies, indicating that reproduction may be by apomixis (61, 601). Likewise, in the F_1 of *P. urvillei* \times *P. malacophyllum* and in backcrosses of the F_1 to *P. urvillei* and *P. dilatatum* reproduction seemed to be predominantly by apomixis. In *P. dilatatum*, Bennett (29) described development of the embryo following fertilization. He did not report, however, observations of fertilization or of a reduced ovule prior to fertilization. The extent of apomixis in this genus needs further investigation.

The possibility of apomixis in *Bouteloua* species should be investigated. Fultz (124) reported somatic chromosome numbers of 21, 28, 35, 42, 61 and 77 in *B. gracilis*, 21, 37 and 42 in *B. hirsuta*, and 40, 42, 45, 56, 70 and 98 in *B. curtipendula*. Such common occurrence of aneuploids and of unequal multiples of the basic number is unexpected in sexually reproducing species.

To explain the origin of apomixis four processes have been proposed—polyploidy, hybridization, genetic factors and necrohormones (522). The most probable explanation seems to be genetic factors conditioning apomixis. Evidence from *Poa* supports this hypothesis (283, 298). In crossing sexual and apomictic forms, only sexual types were obtained (139, 283), and variant type plants (presumably arising from fertilization in most cases) usually produced more variable offspring than the parent from which they arose (7, 283, 298), but numerous exceptions have been found (331). In hybrids of *P. pratensis* \times *P. alpina* the F_1 plants were highly sexual even when apomictic parents were used (7). Similar results have been obtained with F_1 and F_2 plants of *P. compressa* \times *P. pratensis* (304), despite the fact that both parents were highly apomictic (49). These results are contrary to expectation on the hypothesis of hybridity as the cause of apomixis. Müntzing (283) has postulated that apomixis is conditioned by a "balanced constellation" of genes; any disturbance of the balance, as would occur in the occasional functioning of the sexual process, would tend to prevent apomixis.

SUMMARY AND CONCLUSIONS

Cytogenetical investigations of the Gramineae have been initiated primarily for two reasons: (a) to serve as an adjunct to morphological data in studies of taxonomy and phylogeny, and (b) to provide fundamental information for the improvement of species by breeding.

Several investigators, particularly Avdulov (21), have attempted to correlate data on chromosome numbers, size and morphology with morphological, anatomical, ecological and geographical information in development of a natural system of classification. Too little information is available at the present time to permit an accurate statement of the phylogenetic relationships in the Gramineae.

Chromosome numbers have been recorded for 805 species in 142 genera, exclusive of species of *Triticum*, *Aegilops*, *Secale*, *Avena* and *Zea*. In more than half of the species, somatic chromosome numbers are multiples of 7. Multiples of 5 (or 10), 6 (or 12), 8, 9, 11, 13 and 17 occur also. Variations in basic number occur among genera within tribes, among species within some genera and, in a few cases, among collections within species. An extensive aneuploid series has been found in *Stipa*. Interpretation of the data on chromosome numbers is complicated by the fact that errors no doubt occur in both identification of species and determination of chromosome number. Each of the numbers 5, 7 and 12 has been proposed by various investigators as the primary basic number of the grasses, but the evidence is not yet conclusive for any number.

Variations in chromosome size have been reported among major groups, among genera and species, and among plants of the same species. Differences in size, shape and presence of secondary constrictions or satellites occur among individual chromosomes of many species.

Supernumerary chromosomes and centric fragments occur in several species. In *Sorghum purpureo-sericeum* the supernumerary chromosomes persist in the anthers and ovaries but are lost in the radicle prior to seed ripening and in the shoot tissues as the plants reach maturity.

More than two-thirds of the species of Gramineae are polyploid or have one or more polyploid races. In 99 species, races differing in chromosome number have been found. Polyploidy has been an important factor in formation of new species and in extending the range of geographical and ecological adaptation in most genera of the grasses. It has not been, however, important in the origin of major groups, even of genera. Some species are presumed, on the basis of cytogenetical behavior, to be allopolyploids, while a few species behave cytologically like autopolyploids. Critical cytological

and genetical evidence on type of polyploidy is not available for a vast majority of species, including most of the important forage grasses.

Polyploids have been produced experimentally in several species and in *Triticum* × *Agropyron* hybrids, especially by use of colchicine, have been found in twin seedlings in a few species, and have occurred occasionally in untreated material of species and species hybrids. In general, the morphological, physiological and chemical characteristics of the experimental polyploids of grasses are similar to those reported for other plants. The possibilities of induced polyploidy in grass species and interspecific hybrids have not yet been investigated extensively, and this seems to be a fertile field for further study.

Polyhaploids and aneuploids have been found and investigated in a few species, but more extensive experiments of this kind are needed.

Meiosis has been observed in more than 80 species of grasses, but in most cases the reports have been fragmentary and have dealt with only one or a few plants. Multivalent pairing is characteristic of a few species, and occasional multivalents have been observed in plants of several others. In one case, *Briza media*, the association of four chromosomes results from a reciprocal translocation. In some species the multivalents presumably result from autosyndesis.

Aside from multivalent formation, the most commonly reported irregularity has been unpaired chromosomes at diakinesis and metaphase I that lag and divide equationally at anaphase I or, in some species, are distributed at random to the poles. Daughter half chromosomes from lagging univalents usually are lost in the first or second division of meiosis.

Cases of failure of spindle and of non-homologous pairing in prophase of meiosis have been reported in some forage grasses.

Studies of meiosis in species that behave cytogenetically like autopolyploids and in induced autotetraploids have revealed that multivalent frequency is not always a reliable criterion of the meiotic regularity of an autopolyploid. Three major types of meiotic irregularity in autopolyploids have been found, namely, (a) unequal disjunction of members of the multivalents, (b) incomplete disjunction of the multivalents, resulting in lagging and

dividing univalents at anaphase I, and (c) unpaired chromosomes at metaphase I. In *Dactylis glomerata* quadrivalent frequency was not correlated with fertility, but incidence of univalents at metaphase I accounted for about one-half of the heritable variations in fertility among inbred plants. In this species inbreeding resulted in striking increases in frequency of univalents at metaphase I and in decreases in fertility.

Inversion hybridity has been found commonly in the species that have been investigated extensively.

More than 200 interspecific and intergeneric hybrids have been reported in the forage grasses. Species of several genera, particularly *Bromus*, *Lolium*, *Festuca*, *Agropyron* and *Poa*, are involved. Intergeneric hybrids of *Triticum* spp. \times *Agropyron* spp. have been investigated extensively. Further studies of interspecific and intergeneric hybrids may be expected to provide valuable information for use in systematic and phylogenetic analysis of the Gramineae and for improvement of forage grasses by breeding.

Genetic investigations have lagged behind cytological studies in the forage grasses and no information or only fragmentary data on inheritance of qualitative characters are available for a majority of species. In *Sorghum vulgare*, however, the inheritance and linkage relationships of numerous genes have been reported. Heritability of some quantitative characters has been established in several species but no detailed investigation of size inheritance has been reported.

Ecotypes, differing morphologically and physiologically, have developed in natural populations in different geographic regions and under different environmental conditions in the same region.

Apomixis occurs in species of several genera of grasses. Vegetative apomixis has been found in *Festuca*, *Poa* and *Deschampsia*. Agamospermy occurs in several *Poa* species, *Calamagrostis obtusa*, and probably in some species of *Paspalum*. The possibility of apomixis in some *Bouteloua* species is suggested by the common occurrence of aneuploid numbers.

The numerous publications dealing with cytogenetics of grasses are indicative of the research effort devoted to this field. Despite this fact, information is incomplete in every phase of grass cytogenetics that has been investigated. The Gramineae is a large family, and a large proportion of the species are used for forage

at least under some conditions. With so many species awaiting investigation, the cytogenetics of forage grasses presents a problem, the analysis of which has hardly been started.

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TABLE 1

2N CHROMOSOME NUMBERS OF THE GRAMINEAE¹

Subfamily 1. Festucoideae

Tribe 1. Bambuseae*Bambusa*

- Bambusa* sp. 68-74(21)
B. floribunda Zoll. et Mor. 72(550)
B. multiplex Raeusch. 72(550)
B. nana Roxb. 72(596)

Arundinaria

- Arundinaria* sp. 68-74(21)
A. fortunei Fenzl. 48(153)
A. hindsii Munro 48(550)
A. pygmaea Kurz. 54(153)

Phyllostachys

- Phyllostachys* sp. 68-74(21)
P. aurea A. et C. Rivière 48(550)
P. edulis (Carriere) Lehare. 48(cf. 582)
P. heterocyclus Mitford. 48(cf. 582)
P. makinoi Hayata. 48(550)
P. nigra Munro. 48(cf. 582)
P. reticulata Koch. 48(cf. 582)
P. flexusa A. and C. Rivière 54(153)

Sasa

- Sasa kozasa* Nakai. 48(596)
S. kurilensis Nakai 48(596)
S. paniculata Nakai. 48(596)

Sasamorpha

- Sasamorpha borealis* Nakai. 48(596)

Pleioblastus

- Pleioblastus gramineus* (Bean) Nakai. 48(550)
P. maximowiczii Nakai. 48(596)
P. simonii (Carr.) Nakai. 48(550)

Pseudosasa

- Pseudosasa japonica* (Sieb. et Zucc.) Makino. 48(550)

Sasaella

- Sasaella iwatekensis* Makino et Uchida. 48(550)

Semiarundinaria

- Semiarundinaria yashadake* (Makino) Makino. 48(550)

Chimonobambusa

- Chimonobambusa angulata* (Munro) Makino. 48(550)
C. marmorea (Mitford) Makino. 48(550)

Sinobambusa

- Sinobambusa tootsik* (Makino) Makino. 48(550)

Dendrocalamus

- Dendrocalamus strictus* Nees. 72(550)

Tribe 2. Festuceae*Bromus*

- Bromus abolinii* Drob. 14(21)
B. arvensis L. 14(21, 100, 516)
B. brizaeformis Fisch. and Mey. 14(21)
B. dalihonii Trin. 14(21)
B. grandis (Shear) Hitchc. 14(525)
B. intermedius Guss. 14(21)
B. japonicus Thumb. 14(21)
B. kalmii A. Gray. 14(516)
B. laevipes Shear. 14(525)
B. orcuttianus Vasey. 14(525)
B. porteri Nash. (*B. anomalus* Hitchc., non Rupr.) 14(524)
B. purgans L. 14(516)
B. ramosus Huds. 14(516)
B. squarrosus L. 14(100, 516)
B. sterilis L. 14(100, 516)
B. suksdorfii Vasey. 14(526)
B. tectorum L. 14(21, 100, 220, 516)
B. vulgaris (Hook.) Shear. 14(526)
B. adoensis Hochst. 28(100)
B. albidus Bieb. 28(21)
B. anomalus Rupr. 28(330)
B. arenarius Labill. 28(220)
B. australis Spreng. 28(516)
B. grossus Desf. 28(100)

¹ Where necessary and possible, authorities for specific names have been corrected to conform with those given by Hitchcock (150). Likewise, Hitchcock (150) has been used in corrections for synonymy. In cases where the names given by the various authors are listed as synonyms of other valid names, the name listed as valid by Hitchcock (150) has been used. In particularly doubtful cases, the name originally reported is given in parenthesis. When the reported name is not listed in Hitchcock (150) it has been included as in the original report. The author has not attempted to verify identifications by the various investigators and does not assume responsibility for accuracy of the identification.

- B. hordeaceus* L. 28(516)
B. interruptus (Hack.) Druce. 28(268)
B. lepidus Holmb. 28(268)
B. mollis L. 28(21, 219, 220, 516, 525)
B. racemosus L. 28(220, 268)
B. rubens L. 28(27, 220, 516)
B. texensis (Shear) Hitchc. 28(524)
B. cappadocicus Boiss. and Bal. 42(516)
B. macrantherus Hack. 42(100)
B. pacificus Shear. 42(516)
B. pumpellianus Scribn. 42(516)
B. sitchensis Trin. 42(516)
B. trinii Desv. 42(220)
B. uruguayensis Arech. 42(524)
B. variegatus Bieb. 42(516)
B. brevianistatus Buckl. 56(516)
B. arizonicus (Shear) Stebbins 84(527)
B. arduennensis Drun. 14(516); 28(100)
B. macrostachys Desf. 14(516); 28(21, 100)
B. secalinus L. 14(326); 28(21, 100, 220, 314, 516)
B. ciliatus L. 14(516); 56(21)
B. catharticus Vahl. 28(516); 42(21, 526)
B. madritensis L. 28(21, 100, 220); 42(516)
B. commutatus Schrad. 28(220); 56(326)
B. erectus Huds. 42(516)
 var. *eu-erectus* Aschers. et Graebn. 42(516); 56(202)
B. rigidus Roth 42(100); 56(21, 220, 516, 525); 56, 70(27)
 var. *gussonei* Parl. 28(516); 56(100)
B. inermis Leyss. 56(21, 100); 42(516); 56, 70(326); 42, 56(217)
B. carinatus Hook. and Arn. 56(220, 516, 525)
 B. marginatus Nees. 56(21, 27); 42, 70(326, 330)
 B. carinatus hookerianus (Thurb.) Shear. 14(516)
 B. polyanthus Scribn. 42(516)
Brachypodium
 Brachypodium sylvaticum (Huds.) Beauv. 18(21)
 B. pinnatum (L.) Beauv. 14(21)
- Festuca*
Festuca alopecurus Schousb. 14(21)
 F. capillata Lam. 14(73)
 F. geniculata (L.) Cav. 14(21)
 F. granatensis Boss. 14(242)
 F. maritima L. 14(21)
 F. pulchella Huter, Porta and Rigo. 14(516)
 F. spadicea L. var. *genuina* s. var. *aurea* 14(249, 516)
 F. tenuifolia Hort. 14(313)
 F. triflora Desf. 14(242, 516)
 F. amethystina L. 28(516)
 F. elmeri Scribn. and Merr. 28(525)
 F. idahoensis Elmer. 28(525)
 F. mairei St. Y. 28(242, 516)
 F. occidentalis Hook. 28(525)
 F. subuliflora Scribn. 28(268, 524)
 F. viridula Vasey. 28(524)
 F. bromoides L. (*F. uniglumis* Soland.) 42(268)
 F. gigantea (L.) Vill. 42(242, 366)
 F. montana Bieb. 42(242)
 F. silvatica Vill. 42(516)
 F. spectabilis Jan. 42(516)
 F. californica Vasey. 56(525)
 F. varia Haenke. 14(242, 249); 14, 28, 42(516)
 F. myuros L. 14(21); 42(516)
 F. danthonii Aschers. and Graebn. 28(21)
 var. *inberbis* (Vis.) Aschers. and Graebn. 42(21)
 F. elatior L. 14(73, 202, 249, 330, 533); 14, 42, 70(242); 14, 28, 42(516); 14, 42(113, 313, 314)
 F. rubra L. 42, 56, 70(242); 14, 42(516); 42(71, 120, 268, 313)
 F. ovina L. 14(249); 14, 28, 42, 70(242); 14, 28, 42(516); 56(71); 14, 21, 28, 42(558, 559); 28(314); 14, 21, 28, 49(120); 42(71, 268, 313)
- Scleropoa*
 Scleropoa rigida (L.) Griseb. 14(21, 268, 516)
- Puccinellia*
 Puccinellia lemmonii (Vasey) Scribn. 14(77)
 P. vahlana (Liebm.) Scribn. et Merr. 14(120)
 P. fasciculata (Torr.) Bicknell. 28(481)

- P. parishii* Hitchc. 28(77)
P. phryganodes (Trin.) Scribn. et Merr. 28(120)
P. rupestris (With.) Fern. and Weath. 42(481)
P. distans (L.) Parl. (Reported as *Atropis distans* Griseb.) 14, 42(21)
P. nuttalliana (Schult.) Hitchc. 42, 56(77)
P. pumila (Vasey) Hitchc. 42(120); 42, 56(77)
P. maritima (Huds.) Parl. 42, 56(77); 63(268)
- Glyceria*
Glyceria erecta Hitchc. 14(75)
G. fernaldii (Hitchc.) St. John (= *G. neogaea* Steud.) 14(75)
G. pauciflora Presl. 14(75)
G. nervata Trin. 28(516)
G. borealis (Nash) Batchelder. 20(75)
G. cookei Swallen. 20(75)
G. elata (Nash) Hitchc. 20(75)
G. striata (Lam.) Hitchc. 20(75)
G. acutiflora Torr. 40(75)
G. leptostachya Buckl. 40(75)
G. melicaria (Michx.) F. T. Hubb. 40(75)
G. obtusa (Muhl.) Trin. 40(75)
G. occidentalis (Piper) J. C. Nels. 40(75)
G. septentrionalis Hitchc. 40(75)
G. canadensis (Michx.) Trin. 60(75)
G. pallida (Torr.) Trin. 14(75); 20(266)
G. grandis S. Wats. 20(75); 28(326)
G. fluitans (L.) R. Br. 28(516); 20, 40(75)
 (reported as *G. declinata*) 20(268)
G. plicata Fries. 28(516); 40(268)
G. maxima (Hartm.) Holmb. 28, 56(516); 56(21); 60(76)
- Sclerochloa*
Sclerochloa dura (L.) Beauv. 14(21)
- Fluminea*
Fluminea festucacea (Willd.) Hitchc. 28(77)
- Pleuropogon*
Pleuropogon californicus (Nees) Benth. 16(77); 14(77)
- P. davayi* L. Benson. 16(77)
P. refractus (A. Gray) Benth. 32(77)
 var. *hooverianus* L. Benson. 16(77)
- Poa*
Poa chaixii Vill. 14(21, 277, 320, 516)
P. exilis (Tomm.) Murb. 14(320)
P. sudetica Haenke. 14(21)
P. supina Schrad. 14(320)
P. trivialis L. 14(7, 18, 21, 516)
P. badensis Haenke. 28(18)
P. bolanderi Vasey. 28(524)
P. bulbosa L. 28(18)
P. cusickii Vasey. 28(525)
P. cuspidata Nutt. 28(52)
P. dimorphantha Murb. 28(320)
P. douglasii Nees. 28(525)
P. iridifolia Hauman. 28(490)
P. lanigera Nees. 28(490)
P. macrantha Vasey. 28(18)
P. maroccana Nannf. 28(251)
P. resinulosa Nees. 28(490)
P. rhizomata Hitchc. 28(524)
P. rianulorum Maire et Trab. 28(251)
P. sibirica Roshw. 28(512)
P. sylvestris A. Gray. 28(52)
P. violacea Bell. 28(516)
P. wolffii Scribn. 28(52)
P. arachnifera Torr. 42(52)
P. balfourii Parnell. 42(268)
P. bodryoides L. 42(18)
P. ochroleuca Stern. 42(18)
P. unilateralis Scribn. 42(524)
P. altaica Trin. 56(512)
P. epilis Scribn. 56(18)
P. nervosa (Hook.) Vasey. 56(524)
P. tibetica Munro. 56(512)
P. abbreviata R. Br. ca. 76(120)
P. annua L. 14, 28(250); 28(18, 21, 202, 320, 516)
P. sterilis Bieb. 28(516); 42(18)
P. nemoralis L. 28(21); 42(18, 516); 28, 42(7)
P. palustris L. 28, 42(21); 42(516); 28(18)
P. alpina L. 32 to 34(18,21); 42(516); 22 to 38(277); ±31(7); 22, 23, 24, 25, 31(139)
 var. *vivipara* L. 33, 44(120)
P. alpigena (E. Fries) Lindm. ca. 77 and 84(120)

- var. *colpodea* (Th. Fries)
 Scholander. 51(120)
 var. *vivipara* (Malmgr.) Scho-
 lander. 42(120)
P. compressa L. 56(18, 516); 42
 (18, 21); 35, 42, 49(7)
P. glauca Vahl. 42(516); 45
 (202); ca. 70 to 72(120); 70
 (21); 65(7)
P. pratensis L. 56(516); 28, 56,
 70(21); 49 to 85(277); 50 to
 87±1(18); 66, 67(377); 41±
 to 64(52); 48 to 72(508); 28
 to 114(7); 18, 40, 42, ±72
 (215)
P. arctica R. Br. 56(120); 70
 (18)
P. ampla Merr. 62, 64(18)
P. nevadensis Vasey. 64 to 66
 (525); 62(18)
P. scabrella (Thurb.) Benth. 84
 (18); 63, ca. 66, ca. 84, ca. 86
 (525)
P. secunda Presl. 82, ca. 84, 86
 (525)
Briza
Briza minor L. 10(21)
B. elatior Sibth. and Sm. 14(21)
B. maxima L. 14(21)
B. media L. 14(202); 14, 28
 (478)
Desmazeria
Desmazeria sicula (Jacq.) Dum.
 14(21)
Eragrostis
Eragrostis aspera Nees. 20(21)
E. cambessediana Kunth. 20
 (137)
E. cilianensis (All) Link. 20
 (21)
E. tenella (L.) Beauv. 20(21)
E. abyssinica (Jacq.) Link. 40
 (21)
E. albida Hitchc. 40(137)
E. capensis (Lk.) Jedw. 40(21)
E. curvula (Schr.) Nees. 40
 (326)
E. sessilispica Buckl. 40(326)
E. spectabilis (Pursh) Steud. 40
 (326); 42(330)
E. glomerata (Walt) L. H.
 Dewey (Reported as *Poa*
conferta) 56(18)
E. mexicana (Hornem.) Link.
 60(21)
E. pallescens Hitchc. 80(137)
Catabrosa
Catabrosa aquatica (L.) Beauv.
 20(21)
Molinia
Molinia caerulea (L.) Moench.
 36(502)
Distichlis
Distichlis spicata (L.) Greene.
 40(525)
D. stricta (Torr.) Rybd. 40
 (525)
Uniola
Uniola latifolia Michx. 48(21)
Dactylis
Dactylis aschersoniana Graebn.
 14(202)
D. glomerata L. 28(71, 105)
 var. *hispanica* Koch. 28(21)
Cynosurus
Cynosurus balauseae Coss. and
 Dur. 14(21)
C. cristatus L. 14(21, 516)
C. echinatus L. 14(21, 516)
Lamarckia
Lamarckia aurea (L.) Mönch.
 14(21)
Arundo
Arundo donax L. >100(21);
 110(153)
Gynerium
Gynerium sp. 72(22)
G. argentea (Nees) Stapf. 76
 (153)
Ampelodesmos
Ampelodesmos mauretanicus
 (Poir.) Dur. et Schinz. 96
 (524)
Phragmites
Phragmites communis Trin. 36
 (549); 48(21, 153); 96(21)
Melica
Melica altissima L. 18(21, 153,
 202)
M. aristata Thurb. 18(525)
M. californica Scribn. 18(525)
M. ciliata L. 18(21)
M. fugax Boland. 18(525)
M. hyalina Doll. 18(524)
M. imperfecta Trin. 18(525)
M. macra Nees. 18(524)
M. micrantha Boiss. 18(21)
M. mutans L. 18(21, 202)
M. papilionacea L. 18(524)
M. sarmentosa Nees. 18(524)
M. stricta Boland. 18(525)
M. subulata (Griesb.) Scribn. 18
 (525)

- M. torreyana* Scribn. 18(525)
M. uniflora Retz. 18(595)
Schizachne
Schizachne purpurascens (Torr.) Swallen. 20(55)
Triodia
Triodia flava (L.) Smyth. 28(330)
T. thomsonii Petrie. 48(63)
Sieglingia
Sieglingia decumbens Bernh. 36(595); 124(268)
Orcuttia
Orcuttia californica Vasey. var. *inaequalis* Hoover. 24(77)
O. greenii Vasey. 24(77)
O. tenuis Hitchc. 26(77)
O. pilosa Hoover. 32(77)
Neostaffia
Neostaffia colusana Davy. 42(77)
Aeluropus
Aeluropus littoralis (Gorran) Parl. 60(22)
 var. *dasyphylla* Trautv. 20(22)
Boissiera
Boissiera bromoides Hochst. 28(516)
 Tribe 3. *Hordeae*
Agropyron
Agropyron aegilopoides Drob. 14(21)
A. dagnae Grossh. 14(21, 365)
A. prostratum (Pall.) Beauv. 14(21)
A. villosum Link. 14(365)
A. caninum (L.) Beauv. 28(21, 365, 502)
A. ciliare Franch. 28(316)
A. dasystachyum (Hook.) Scribn. 28(365)
A. desertorum (Fisch.) Schult. 28(365)
A. elongatiforme Drob. 28(576)
A. japonicum Honda. 28(316)
A. mutabile Drob. 28(21)
A. orientale (L.) R. and S. 28(21)
A. parishii Scribn. and Sm. 28(524)
A. pringlei (Scribn. and Sm.) Hitchc. 28(524)
A. sibiricum (Willd.) Beauv. 28(21, 365)
A. subsecundum (Link.) Hitchc. 28(365, 588)

- A. trachycaulum* (Link.) Malte. 28(21, 330, 365, 525)
A. tunguscense Drob. 28(21)
A. violaceum var. *major* Vasey. 28(330)
A. griffithsii Scribn. and Smith. 28(365)
A. glaucum R. and S. 42(365)
A. obtusiusculum Lange. 42(365)
A. pungens (Pers.) Roem. and Schult. 42(365)
A. rasemiphyllum Koidz. 42(316)
A. trichophorum (Link) Richt. 42(15)
A. campestre Gren. et Godr. 56(502)
A. spicatum (Pursh.) Scribn. and Smith. 28(475); 14(365)
A. intermedium (Host.) Beauv. 28, 42(208); 42(15, 502)
A. junceum (L.) Beauv. 28(365); 28, 42(356, 503); 42(576)
A. semicostatum (Steud.) Nees. 28(330); 42(316)
A. cristatum (L.) Beauv. 14(502); 14, 28(365); 28, 42(15); 28(21)
A. repens (L.) Beauv. 28, 42(21); 42(365, 502, 533)
A. smithii Rydb. 56(365); 42(524)
 var. *molle* (Scribn. and Smith) Jones. 28, 56(365)
A. elongatum (Host.) Beauv. 14, 70(502); 70(365); 56(486)
Elymus
Elymus caput-medusae L. (= *Hordeum caput-medusae* Coss. and Dur.) 14(135)
E. ambiguus var. *strigosus* (Rydb.) Hitchc. 28(330)
E. europaeus L. 28(595)
E. giganteus Vahl. 28(21)
E. glaucus Buckl. 28(525)
E. macounii Vasey. 28(326)
E. mollis Trin. 28(524)
E. sibiricus L. 28(21, 326)
E. villosus Muhl. 28(330)
E. virescens Piper. 28(524)
E. virginicus L. 28(21, 330)
E. dahuricus Turcz. 42(21)
E. arenarius L. 56(512, 516)

- E. canadensis* L. 28(21, 326, 516); 28, 42(330)
E. triticoides Buckl. 28, 42(525)
E. condensatus Presl. 28, 56(525); 28(330)
- Sitanion**
Sitanion hansenii (Scribn.) J. G. Smith. 28(525)
S. hystrix (Nutt.) J. G. Smith. 28(79, 525)
S. jubatum J. G. Smith. 28(525)
- Hystrix**
Hystrix patula Moench. 28(21)
H. californica (Boland.) Kuntze. 56(524)
- Hordeum**
Hordeum chilense Brongn. 14(14)
H. compressum Gris. 14(363)
H. marinum Huds. (= *H. maritimum* With.) 14(131, 135)
H. pusillum Nutt. 14(14, 70, 363)
H. spontaneum Koch. 14(135, 533)
H. stenostachys Godrn. 14(363)
H. vulgare L. 14(135)
H. bulbosum L. 28(31, 70, 131, 516)
H. gussoneanum Parl. 28(70)
H. leporinum Link. 28(363)
H. secalinum Schreb. 28(131, 516)
H. silvaticum Huds. 28(516)
H. jubatum L. ca. 14(54); 28(2, 70, 135, 516, 363, 525)
H. murinum L. 28(14, 70, 135, 516); 14(131, 363, 533)
H. nodosum L. 14(541); 14, 28(70, 525); 42(135)
- Lolium**
Lolium loliaceum Hand.-Maz. 14(183)
L. multiflorum Lam. 14(113)
L. perenne L. 14(113, 116, 313)
L. persicum Boiss. 14(116)
L. remotum Schrank. 14(116, 183)
L. rigidum Gaudin. 14(183)
L. subulatum Vis. 14(312)
L. temulentum L. 14(116, 179)
- Nardus**
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- Lepturus**
Lepturus filiformis (Roth.) Trin. 14(21)
L. pannonicus Kunth. 14(21)
- L. incurvatus* (L.) Trin. 36(21)
L. cylindricus Trin. 52(153); 26(21)
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Schismus calicinus Coss. 12(21)
- Koeleria**
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K. panicea (Lam.) Dom. 14(21)
K. phleoides (Vill.) Pers. (Reported as *Lophochloa phleoides* Rchbch.) 26(21)
K. vallesiana Aschers. et Graebn. 42(268)
K. cristata (L.) Pers. 28(525, 268); 70(21)
- Sphenopholis**
Sphenopholis intermedia (Rydb.) Rydb. 14(330)
- Trisetum**
Trisetum sibiricum Rupr. 14(21)
T. flavens (L.) Beauv. subsp. *pratense* (Pers.) Beck. 24(21)
T. projectum Louis-Marie 28(524)
T. spicatum (L.) Richt. 28(120) var. *congonii* Hitchc. 28(524)
T. canescens Buckl. 42(525)
T. cernuum Trin. 42(524)
- Deschampsia**
Deschampsia setacea Hack. 14(138, 268)
D. danthonioides (Trin.) Munro. 26(524)
D. elongata (Hook.) Munro. 26(524)
D. holciformis Presl. 26(524)
D. arctica Merr. 28(138)
D. bottnica (Wahl.) Hartm. 28(138)
D. caespitosa (L.) Beauv. 28(21, 138, 330)
D. flexuosa (L.) Trin. 28(138, 516)
D. pumila Nees. 28(138)
D. alpina Roem. 39, 41, 49(120); 56(138)
- Aira**
Aira caryophyllea L. 14(138, 594)
A. praecox L. 14(138, 268)
A. multiculmis Dum. 28(138)
- Arrhenatherum**
Arrhenatherum elatius (L.) Mert. and Koch. 28(2, 314)

- var. *bulbosum* (Willd.) Spenner. 28(481)
- A. avenaceum* Beauv. = *A. elatius* (L.) Mert. and Koch. ca. 40 (105)
- Holcus*
- Holcus lanatus* L. 14(21, 516)
- H. mollis* L. 14(516)
- Danthonia*
- Danthonia carphoides* F. Muell. 24(524)
- D. duttoniana* Cashmore. 24 (524)
- D. gracilis* Hook. f. 24(63)
- D. nigricans* (Petrie) Calder. 24 (63)
- D. purpurea* Beauv. 24(524)
- D. setifolia* (Hook. f.) Calder 24(63)
- D. australis* J. Buch. 36(63)
- D. californica* Boland. 36(525)
- D. chilensis* Desv. 36(524)
- D. compressa* Austin. 36(524)
- D. crassiuscula* Kirk. 36(63)
- D. intermedia* Vasey. 36(524)
- D. oreophila* Petrie. 36(63)
- D. spicata* (L.) Beauv.
- var. *typica* Fernald. 36(524)
- D. unispicata* Munro. 36(525)
- D. cunninghamii* Hook. f. 42(63)
- D. ovata* J. Buch. 42(63)
- D. raoulii* Steud. 42(63)
- D. richardsonii* Cashmore. 48 (524)
- D. buechanani* Hook. f. 72(63)
- D. pilosa* Hook. f. 24(524); 48 (63)
- D. semiannularis* Hook. f. 24 (524); 48(63)
- Dupontia*
- Dupontia fisherii* R. Br. 88(120)
- var. *psilosantha* Rupr. 44(120)
- Tribe 5. Agrostideae**
- Calamagrostis*
- Calamagrostis arundinacea* (L.) Roth. 28(21)
- C. koelerioides* Vasey. 28(524)
- C. nutkaensis* (Presl) Steud. 28 (524)
- C. rubescens* Buckl. 28(524)
- C. breweri* Thurb. 42(524)
- C. canadensis* (Michx.) Beauv. 56(524)
- C. epigeios* (L.) Roth. ca. 70 (21); 28(326)
- C. neglecta* (Ehrh.) Gaertn.
- Mey. and Schreb. ca. 70(21); 28(120)
- Ammophila*
- Ammophila arenaria* (L.) Link. 28(594)
- A. breviligulata* Fernald. 28(71)
- Agrostis*
- Agrostis biebersteiniana* Claus. 14(511)
- A. interrupta* L. 14(268, 511)
- A. nebulosa* Boiss. and Reut. 14 (21, 511, 546)
- A. perennans* (Walt.) Tuckerm. (Reported as *A. elegans* Thore) 14(511)
- A. setacea* Vill. 14(268)
- A. mongholica* Roshev. 28(511)
- A. rossae* Vasey. 28(524)
- A. tenuis* Sibth. 28(21, 511)
- A. verticillata* Vill. 28(21, 511)
- A. prorepens* G. Mey. 35(511)
- A. clavata* Trin. 42(511)
- A. diegoensis* Vasey. 42(525)
- A. gigantea* Roth. 42(511)
- A. hallii* Vasey. 42(525)
- A. hiemalis* (Walt.) B.S.P. 42 (511)
- A. hissarica* Roshev. 42(511)
- A. nigra* With. 42(268)
- A. planifolia* C. Koch 42(511)
- A. borealis* Hartm. 56(511, 512)
- A. lepida* Hitchc. 56(524)
- A. retrofracta* Willd. 56(524)
- A. canina* L. 14(511); 28(594)
- A. trinii* Turcz. 28(478); 14, 28 (511)
- A. alba* L. 28(330, 511); 42(21, 508)
- A. exarata* Trin. 28(326); 42 (525)
- A. palustris* Huds. 28(73)
- A. palustris* Huds. [Reported as *A. alba* var. *maritima* (Lam.) Meyer] 56(73)
- Phippsia*
- Phippsia algida* (Soland.) R. Br. 28(120)
- P. concinna* (Th. Fr.) Lindeb. 28(120)
- var. *algidiformis* (Smith) Holmb. 29(120)
- Mibora*
- Mibora verna* Beauv. 14(21)
- Alopecurus*
- Alopecurus aequalis* Sobol. 14 (202, 534)
- A. amurensis* Kom. 14(534)

- A. bulbosus* (L.) Poir. 14(268)
A. longearistatus Maxim. 14 (534)
A. myosuroides Huds. 14(71, 202, 534)
A. mucronatus Hack. 28(512, 534)
A. pratensis L. 28(21, 71, 202, 263, 512, 534)
A. seravachanicus Ovcz. 28(534)
A. soongoricus V. Petr. 28(534)
A. dasyanthus Trautv. 56(534)
A. glacialis C. Koch. 56(534)
A. himalaicus Hook. 56(534)
A. textilis Boiss. 56(534)
A. vaginatus Pall. 56(512, 534)
A. pseudobrachystachus Ovcz. 98 (534)
A. glaucus Lees. 130(512)
A. geniculatus L. 14(202); 28 (21, 512, 534)
 var. *aristulatus* Torr. 14(71)
A. antarcticus Vahl. 112 to 116 (188)
A. alpinus J. E. Smith 119 to 122 (188); 112, 114, ± 130(120)
 (Reported as *A. borealis* Trin.) 98(534)
A. alpinus var. *elatius* Kom. 70 (21)
Polypogon
Polypogon monspeliensis (L.) Desf. 28(21)
P. lutosus (Poir.) Hitchc. 28 (481)
 [Reported as *P. littoralis* (With.) Smith f. *gracilis*] 42(21)
Phleum
Phleum boehmeri Wibel. 14(21)
P. michelii All. 14(202)
P. phleoides L. 14(153)
P. subulatum (Savi) Aschers. and Graebn. 14(292)
P. paniculatum Huds. 28(21)
P. alpinum L. 14, 28(134)
P. pratense L. 14, 42(134); 42 (21, 508)
Gastridium
Gastridium ventricosum Schinz. and Thell. 14(481)
G. ventricosum Schinz. and Thell. (Reported as *Alopecurus ventricosum* Pers.) 28 (534)
Lagurus
Lagurus ovatus L. 14(21)
- Muhlenbergia**
Muhlenbergia filiformis (Thurb.) Rydb. 18(524)
M. andina (Nutt.) Hitchc. 20 (524)
M. asperifolia (Nees and Mey.) Parodi. 20(524)
M. mexicana (L.) Trin. 40(21)
M. racemosa (Michx.) B.P.S. 40(21)
M. rigens (Benth.) Hitchc. 40 (525)
M. squarrosa (Trin.) Rydb. 40 (525)
M. sylvatica Torr. 40(21)
M. pungens Thurb. 42(330); 60 (326)
Sporobolus
Sporobolus cyptandrus (Torr.) A. Gray. 18(326)
S. diander Beauv. 36(21)
S. poiretii (Roem. and Schult.) Hitchc. 36(21)
S. tenuissimus Kuntze. 40(153)
S. heterolepis A. Gray. 72(326)
S. airoides (Torr.) Torr. 126 (525)
S. indicus (L.) R. Br. 18, 36 (21)
Crypsis
Crypsis aculeata (L.) Ait. 54 (524)
Heleochoa
Heleochoa schoenoides (L.) Host. 36(21)
Milium
Milium vernale Bieb. 18(21)
M. effusum L. 28(21)
Oryzopsis
Oryzopsis miliacea (L.) Benth. and Hook. 24(21)
O. virescens Beck. 24(21)
O. hymenoides (Roem. and Schult.) Ricker. 48(185, 525)
Stipa
Stipa sibirica Lam. 24(21, 256a)
S. leucotricha Trin. and Rupr. 28(256a)
S. neesiana Trin. and Rupr. 28 (524)
S. pinetorum Jones 32(524)
S. lemmoni (Vasey) Scribn. 34 (256a)
 var. *jonesii* Scribn. 34(525)
S. lepida Hitchc. 34(525)
S. megapotamica Sprengel 34 (524)

- S. thurberiana* Piper. 34(525)
S. californica Merr. and Davy. 36(525)
S. elmeri Piper and Brodie. 36(525)
S. mucronata H. B. K. 36(256a)
S. occidentalis Thurb. 36(525)
S. philippii Steud. 36(524)
S. coronata Thurb. 40(525)
S. stillmanii Boland. 40(524)
S. pringlei Scribn. 42(256a)
S. humilis Cav. 42 [-44] (524)
S. columbiana Macoun. 44(326)
S. juncea L. 44(21, 256a)
S. lessingiana Trin. and Rupr. 44(21)
S. loannis Cel. 44(21)
S. neomexicana (Thurb.) Scribn. 44(256a)
S. papposa Ness. 44(21)
S. pulcherrima C. Koch. 44(21)
S. stenophylla Czern. 44(21)
S. ucrainica P. Smirn. 44(21)
S. eminens Cav. 46(256a)
S. spartea Trin. 46(256a)
S. splendens Trin. 48(256a)
S. robusta Scribn. 64(256a)
S. lettermani Vasey. 68(256a)
S. speciosa Trin. and Rupr. 68(256a)
S. cernua Stebbins and Love. 70(525)
S. viridula Trin. 82(185)
S. brachychaeta Godr. 40(490); 44 to 46(524)
S. comata Trin. and Rupr. 44 to 46(525); 46(256a)
 (Reported as *S. capillata* L.) 44(21)
S. pulchra Hitchc. 66(326); 64(525)
Aristida
Aristida hamulosa Henr. 44(525)
A. adscensionis L. 22(22)
Arctagrostis
Arctagrostis latifolia (R. Br.) Griseb. 62(120)
Arctophila
Arctophila fulva (Trin.) Rupr. 42(120)

Tribe 6. Zoysieae

- Tragus*
Tragus racemosus (L.) All. 40(21)
Antheophora
Antheophora hermaphrodita (L.) Kuntze. 18(21)

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- Leptochloa*
Leptochloa polystachya Benth. 20(21)
Eleusine
Eleusine indica (L.) Gaertn. 18(21, 224, 226)
E. oligostachya Link. 18(224)
E. tristachya Lam. 18(21)
E. brevifolia Hochst. 36(224, 226)
E. coracana (L.) Gaertn. 36(21, 153, 224, 226)
E. flagellifera Nees. 45(224)
Dactyloctenium
Dactyloctenium aegyptium (L.) Richt. (Reported as *Eleusine aegyptiaca* Desf. et Cormbatore) 34(226)
D. aegyptium (L.) Richt. 48(21)
D. scindicum Boiss. 48(224)
Cynodon
Cynodon dactylon (L.) Pers. 30(153); 36(21)
Beckmannia
Beckmannia eruciformis Host. 14(21)
B. syzigachne (Steud.) Fernald. 14(330)
Spartina
Spartina schreberi J. F. Gmel. 40(21)
S. bakeri Merr. 42(73)
S. gracilis Trin. 42(73)
S. spartinae (Trin.) Merr. 42(74)
S. leiantha Benth. 56(74)
S. stricta Roth. 56(154, 155)
S. townsendii H. and J. Groves. 126(154, 155)
S. patens (Ait.) Muhl. 42(73); 28(74)
 var. *juncea* (Michx.) Hitchc. 42, 56(74); 28(73)
S. alterniflora Lois. 56, 70(74); 70(154, 155)
S. cynosuroides (L.) Roth. 42(73, 74); 80(21)
S. pectinata Link. 42, 84(74)
 (Reported as *S. michauxiana* Hitch.) 28(71)

Chloris

- Chloris distichophylla* Lag. 20(224)
C. gracilis 30(224)
C. acuminata Trin. 40(21)
C. caudata Trin. 40(224)

- C. cucullata* Bisch. 40(21)
C. truncata R. Br. 40(21, 224)
C. bournei Rang. et Tad. 50(224)
C. virgata Swartz. 14(330); 30(224)
C. barbata (L.) Sw. 40(224); 20(21)
C. gayana Kunth. 20(21, 153); 20, 40(271)
C. submutica H. B. K. 80(21); less than 65(224)
- Trichloris*
Trichloris mendocina (Phil.) Kurtz. 40(21)
- Bouteloua*
Bouteloua brevisetata Vasey. 21(124)
B. eriopoda (Torr.) Torr. 21(124)
B. rothrockii Vasey. 22(124)
B. rigidisetata (Steud.) Hitchc. 35(124)
B. hirsuta Lag. 21, 37, 42(124)
B. curtipendula (Michx.) Torr. 40, 42, 45, 56, 70, 98(124); 42(330)
B. gracilis (H. B. K.) Lag. 40(21); 42(330); 21, 28, 35, 42, 61, 77(124)
- Buchloë*
Buchloë dactyloides (Nutt.) Engelm. 56(326); 60(21)
- Dinebra*
Dinebra retroflexa (Vahl.) Parz. 20(21)
- Tribe 8. *Phalarideae*
Hierochloë
Hierochloë occidentalis Buckl. 42(77)
H. alpina (Swartz) Roem. 56(77)
H. odorata (L.) Beauv. 42(21); 28, 56(77)
- Anthoxanthum*
Anthoxanthum aristatum Boiss. 10(21, 269, 355)
A. ovatum Lag. 10(355)
A. amarum Brot. 80(355)
A. odoratum L. 20(21, 153, 269, 359); 10, 20(355)
- Phalaris*
Phalaris brachystachys Link. 12(269, 359)
P. angusta Nees. 14(490)
P. lemmonii Vasey. 14(359)
P. paradoxa L. 14(269, 359)
- P. californica* Hook. and Arn. 28(525)
P. minor Retz. 28(21, 269, 359)
P. tuberosa L. 28(71, 182, 269, 330, 359)
P. canariensis L. 12(21, 71, 202, 269, 359, 490); 28(315)
P. arundinacea L. 28(21, 153, 182, 269, 313, 330, 359); 14, 28(71)
P. coerulescens Desf. 14(359, 490); 28(269)
- Ehrharta*
Ehrharta erecta Lam. 24(359)
E. calycina Sm. 48(359)
E. longiflora Sm. 48(359)
E. panicacea Sm. 24, 48(502)
- Microlaena*
Microlaena stipoides R. Br. 48(359)
- Tribe 9. *Oryzeae*
Oryza
Oryza barthii Cheval. 24(376, 550)
O. glaberrima Steud. 24(376)
O. longistaminata Cheval. et Roehrich. 24(376)
O. meyeriana Baillon. 24(550)
O. officinalis Wall. 24(376, 550)
O. sativa L. 24(376, 550)
O. minuta J. et C. Presl. 48(376, 550)
O. latifolia Desv. 48(376); 24, 48(550)
- Leersia*
Leersia hexandra Swartz. 48(376, 550)
 "L. japonica" Makino. (Name never properly published) 96(550)
L. oryzoides (L.) Swartz var. *japonica* Hack. 48(376); 60(550)
- Hygoryza*
Hygoryza aristata Nees. 24(376, 550)
- Chikusichloa*
Chikusichloa aquatica Koidz. 24(550)
- Lygeum*
Lygeum spartum Loebl. ex L. 40(376)
- Tribe 10. *Zizanieae*
Zizania
Zizania aquatica L. 30(376)
Z. latifolia Turcz. 30(376)

*Zizaniopsis**Zizaniopsis miliacea* (Michx.)

Doell and Ascher. 24(54)

Subfamily 2. *Panicoideae*Tribe 11. *Melinideae**Melinis**Melinis minutiflora* Beauv. 36

(21, 153)

Tribe 12. *Paniceae**Trichachne**Trichachne californica* (Benth.)Chase. (Reported as *Panicum**californicum*) 18(224)*Digitaria**Digitaria foliosa* Lag. (= *Paspalum vaginatum* Swartz) 16

(597)

D. valida var. *glauca* Stent. 24

(597)

D. sanguinalis (L.) Scop. 28

(72); 36(224)

D. decumbens Stent. 30(60)*D. polevansii* Stent. 34(60)*D. pentzii* Stent. 36(60)*D. exilis* Stapf. 54(153)*Eriochloa**Eriochloa villosa* Kunth. 54(21)*Brachiaria**Brachiaria mutica* Stapf. 36(153)*Axonopus**Axonopus affinis* Chase. 80(60)*Paspalum**Paspalum ciliatifolium* Michx. 20

(59)

P. gayanus Desv. 20(490)*P. haumanii* Parodi. 20(489)*P. paniculatum* L. (= *P. repens* Bergius) 20(59)*P. pubescens* Muhl. (Reported as*P. muhlenbergii* Nash.) 20

(72)

P. stoloniferum Bosc. 20(21,

263)

P. supinum Bosc. 20(60)*P. alnum* Chase. 24(60)*P. dissectum* (L.) L. (Reported as *P. membranaceum*) 25

(224)

P. boscianum Flügge. 40(59)*P. commune* (Hackel) Lillo, Emend. Parodi. 40(489)*P. cromyorrhizon* Trin. 40(489)*P. hieronymi* Hackel. 40(489)*P. intermedium* Munro. 40(60,

490)

P. lanceolatum Mikan. 40(224)*P. malacophyllum* Trin. 40(59)*P. notatum* Flügge. 40(59)*P. plicatum* Michx. 40(489)*P. proliferum* Arech. 40(490)*P. scrobiculatum* L. 40(21)*P. simplex* Morong. 40(489)*P. unispicatum* (Scribn. and Merr.) Nash. 40(489)*P. lentiferum* Lam. 45(224)*P. setaceum* Michx. 50(224)*P. tenellum* Willd. 55(224)*P. langei* (Fourn.) Nash. 60

(60)

P. longipilum Nash. 60(60)*P. virgatum* L. 80(21)*P. epilis* Parodi. 80(489)*P. giganteum* Baldw. 120(60)*P. floridanum* Michx. 160(59)*P. quadrifarium* Lam. 60(224);

30(60); 20(489)

P. dilatatum Poir. 40(59, 263);

50(224)

P. urvillei Steud. 40, 60(326);

40(59)

P. distichum L. 48(60); 60(489)*Panicum**Panicum antidotale* Retz. 18(60)*P. arenicoloides* Ashe. 18(60)*P. capillare* L. 18(21)*P. commutatum* Schult. 18(60)*P. eruciforme* J. E. Sm. 18(21,

224)

P. lindheimeri Nash. 18(72, 326)*P. scribnerianum* Nash. 18(72)*P. sphaerocarpon* Ell. 18(72)*P. subvillosum* Ashe. 18(72)*P. tsugetorum* Nash. 18(72)*P. tuberculatum* Presl. 18(224)*P. acroanthum* Steud. 36(21)*P. distachyum* L. 36(224)*P. fasciculatum* Swartz. 36(60)*P. miliare* Lam. 36(470)*P. paludivagum* Hitchc. and

Chase. 36(60)

P. purpurascens Raddi. 36(60)*P. clandestinum* L. 40(224)✓ *P. repens* L. 40(224)*P. teneriffae* R. Br. 40(224)*P. dichotomiflorum* Michx. 54

(72)

P. jubiflorum Trin. 54(60)*P. esculentum* A. Br. 56?(224)*P. altissimum* 60(224)*P. bulbosum* H. B. K. 70(224)*P. anceps* Michx. 18(326); 36

(60)

P. texanum Buckl. 36(326); 54

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- P. miliaceum* L. 36, 72(16); 42 (470); 36(21); 40 or 36(72)
P. virgatum L. 36, 72(74); 72 (60); 70(224); 18, 36, 54, 72, 90, 108(328)
Sacciolepis
Sacciolepis striata (L.) Nash. (Reported as *Panicum fluitans*) over 100(224)
Oplismenus
Oplismenus undulatifolius (Ard.) Beauv. 54(21)
O. burmanii (Retz.) Beauv. 72 (153)
O. compositus Beauv. 72(21)
Echinochloa
Echinochloa colonum (L.) Link. (Reported as *Panicum colonum*) 130(224)
E. crusgalli (L.) Beauv. 42(72) var. *frumentacea* (Roxb.) Wight. 56(72); 48(470); 36 (153); 54(21)
Tricholaena
Tricholaena rosea Nees. 36(21)
Setaria
Setaria italica (L.) Beauv. 18 (21, 216, 225, 313)
S. viridis (L.) Beauv. 18(21, 216)
S. faberii Herrm. 36(216)
S. lutescens (Weigel) F. T. Hubb. 36(21, 216, 225)
S. palmifolia (Willd.) Stapf. 36 (21)
S. geniculata (Lam.) Beauv. 72 (216)
S. verticillata (L.) Beauv. 36 (21); 18(225); 20(224)
Pennisetum
Pennisetum compressum R. Br. 14(224)
P. glaucum (L.) R. Br. 14(21, 60, 224)
P. ruppellii Steud. 27(21)
P. purpureum Schumach. 28(60)
P. cenchroides Rich. 36(21)
P. clandestinum Hochst ex Chiov. 36(153)
P. longistylum Hochst. 45(21)
P. villosum R. Br. 45(21)
P. macrourum Trin. (= *P. macrurum*) 54(21, 224)
P. setosum (Swartz) L. Rich. 54(21)
P. orientale var. *triflorum* (Nees) Stapf. 45(224)
P. orientale Rich. 36(21)
Cenchrus
Cenchrus echinatus L. 34(21)
C. inflexus R. Br. 34(21)
C. tribuloides L. 34(21, 153)
C. myosuroides H. B. K. 70(21)
Tribe 13. Andropogoneae
Imperata
Imperata cylindrica (L.) Beauv. 20(cf. 21, 42, 165)
Miscanthus
Miscanthus japonicus Anderss. 36(21)
M. sinensis Anderss. 42(72, 153)
M. saccharifera Benth. 64(153)
Saccharum
Saccharum ciliare Anderss. 40 (42)
S. officinarum L. 80(41, 165, 166)
S. robustum Brandes. 80(165)
S. spontaneum L. 112(41); 48, 56, 64, 72, 80, 96, 112(165)
Erianthus
Erianthus arundinaceus (Retz.) Jeswiet. 60(42)
E. japonicus Beauv. 60(42)
E. sara (Roxb.) Rümke. 60 (165)
E. ravennae (L.) Beauv. 20 (165); 60(42)
Arthraxon
Arthraxon ciliaris Beauv. subsp. *langsдорffii* (Trin.) Hack. 36 (21)
A. hispidus Merr. 36(21)
Andropogon
Andropogon elliotii Chapm. 20 (73, 153)
A. glomeratus (Walt.) B. S. P. 20(73)
A. micranthus Kunth. 20(74)
A. monticola Schult. 20(224)
A. pumilus Roxb. 20(224)
A. virginicus L. 20(73, 74)
A. caesioides Nees. 22(cf. 224)
A. annulatus Forsk. 40(198)
A. distachyum L. 40(490)
A. gryllus L. 40(21)
A. guianensis Steud. 40(224)
A. piptatherus Hack. 40(21)
A. scoparius Michx. 40(73, 74, 153, 326)
A. foveolatus Delile. 45(224)
A. ischaemum L. 45(224)
A. caricoides L. 50(224)
A. intermedius R. Br. 60(21)

- A. ternarius* Michx. 60(74)
A. perforatus Trin. 80(74, 326)
A. furcatus Muhl. 60(326); 70(72)
 (Reported as *A. provincialis* Lam.) 40, 60(74)
A. hallii Hack. 60, 70(326); 60(74)
A. saccharoides Swartz. 60, 70(326); 60(21); 100(74)
- Cymbopogon*
Cymbopogon caesius (Nees) Stapf. 20(550)
C. nardus (L.) Rendle. 20(231)
C. polyneuros (Steud.) Stapf. 20(550)
C. coloratus (Hook.) Stapf. 40(550)
C. martini (Roxb.) W. Wats. 40(550)
C. flexuosus (Nees) W. Wats. 20, 40(550)
C. citratus (D. C.) Stapf. 40, 60(550)
- Hyparrhenia*
Hyparrhenia hirta (L.) Stapf. 30(128)
- Sorghum*
 ✓ *Sorghum dimidiatum* Stapf. 10(157, 163, 443)
 ✓ *S. versicolor* Anders. 10(163, 253, 443)
S. arundinaceum (Willd.) Stapf. 20(157, 253)
S. effusum (Hack.) Karper et Chisholm. 20(198)
S. hewisoni (Piper) Karper et Chisholm. 20(198, 253)
S. lanceolatum Stapf. 20(157)
S. verticilliflorum (Stead.) Stapf. 20(157, 198, 253)
S. virgatum (Hack.) Stapf. 20(157, 198, 253)
S. vogelianum (Piper) Stapf. 20(157)
S. vulgare Pers. 20(157, 198, 231, 253, 263, 313, 470)
 var. *sudanense* (Piper) Hitchc. 20(115, 157, 198, 253, 313)
S. alnum Parodi 40(482)
S. nitidum Pers. 10(253, 443); 20(524)
- S. purpureo-sericeum* Aschers. and Schweinf. 10(157, 163, 443); 40(253)
S. halepense (L.) Pers. 40(115, 157, 198, 253, 313); 20(202)
- Sorghastrum*
Sorghastrum elliottii (Mohr) Nash. 20(524)
S. secundum (Ell.) Nash. 20(524)
S. nutans (L.) Nash. 40(72)
- Rotboellia*
Rotboellia glandulosa Trin. 54(21)
- Manisuris*
Manisuris cylindrica (Michx.) Kuntze. 18(262)
- Narenga*
Narenga porphyrocoma (Hance) Bor. (= *N. narenga*) 30(165, 166)
- Ischaemum*
Ischaemum timorense Kunth. (= *Spodiopogon byronis* Trin.) 20(42)
- Tristachya*
Tristachya hispida Schum. 24(524)
- Themeda*
Themeda arguens Hack. 20(21)
T. forskalii Hack. 60(21)
- Apluda*
Apluda mutica L. 20(153)
- Tribe 14. Tripsaceae
Coix
Coix aquatica Roxb. 10(262)
C. lachryma-jobi L. 20(252, 262, cf. 550)
- Tripsacum*
Tripsacum floridanum Porter 36(254)
T. latifolium Hitchc. 72(262)
T. laxum Nash. 72(262)
T. dactyloides (L.) L. 70(252, 330); 36(153); 36, 72(12, 262)
- Euchlaena*
Euchlaena mexicana Schrad. 20(231, 252, cf. 550)
E. perennis Hitchc. 40(252, cf. 550)
- Polytoca*
Polytoca macrophylla Benth. 40(21, 64)

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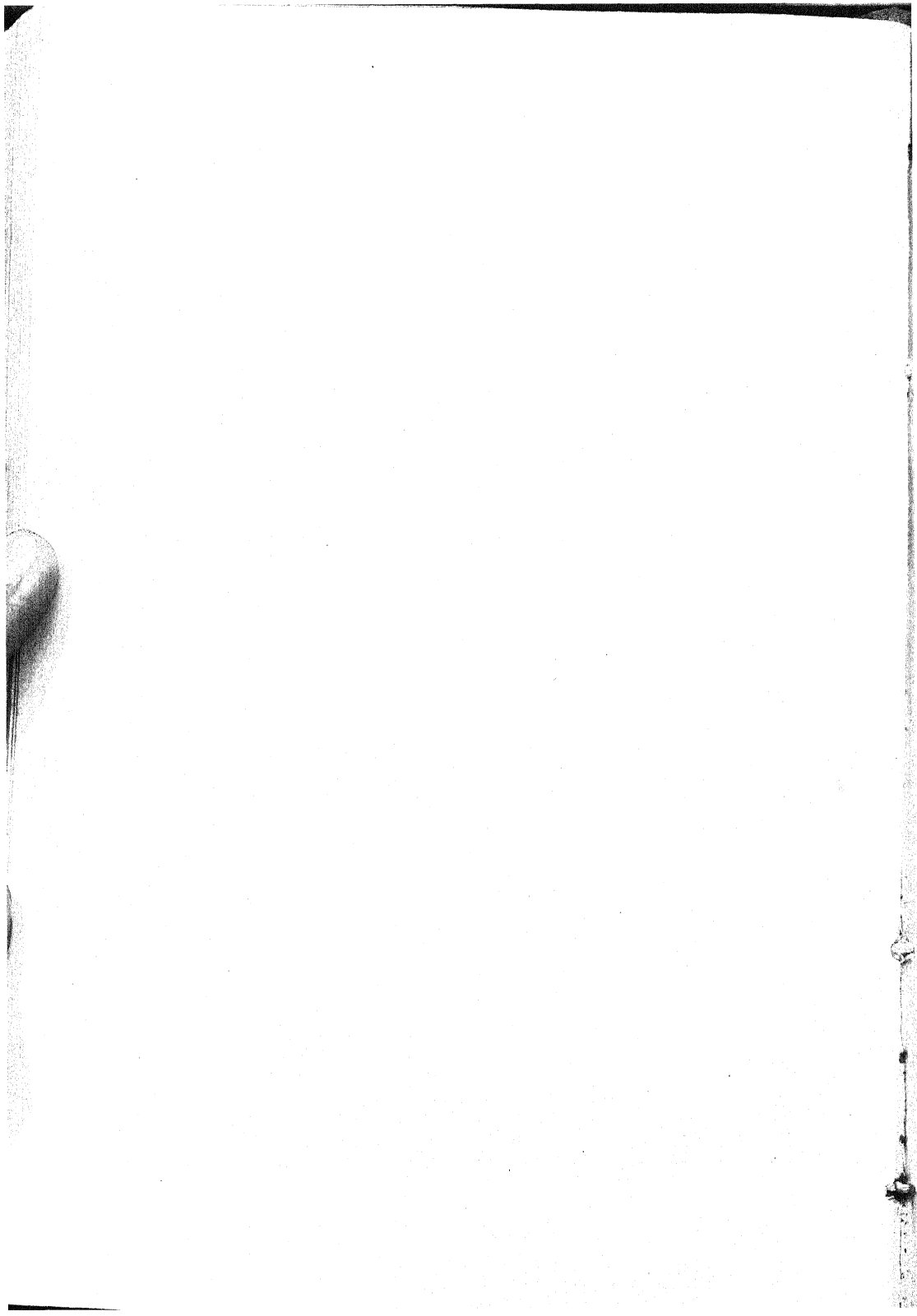
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THE ENDOSPERM IN SEED DEVELOPMENT

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INTRODUCTION

Two fertilizations occur at the inception of the sporophytic generation in flowering plants. The egg, following conjugation with one of the two sperms conveyed to the female gametophyte by the pollen tube, gives rise to the embryo. The central cell of the female gametophyte is fertilized by the other sperm and develops into the endosperm. The latter fertilization involves the union of three nuclei, an antipodal polar, a micropylar polar and a sperm nucleus. Thus not only are both endosperm and embryo of biparental origin in cross-pollinated species but the two structures differ in hereditary organization. The endosperm is $3x$, having received a double complement of inheritance from the pistillate parent. The embryo is $2x$. Genetic diversity within the seed is further increased by the fact that, since the maternal tissues and the embryo belong to different sporophytic generations, they may be unlike in genotype.

The embryo embodies the line of descent and, therefore, is the principal component of the seed. Endosperm, nucellus and integuments are ancillary to the embryo in maintaining the succession. These associated tissues fulfill two general functions. They furnish the medium in which the fertilized egg is transformed into the embryonic sporophyte; and, secondly, they provide a vehicle for the embryo during the interval between maturity and establishment of the seedling.

The significance of the endosperm mainly lies in the fact that it plays a major rôle in the development and maintenance of a medium suitable for growth of the young embryo. Indeed the endosperm appears to have no other function in many species. Often it does

¹ Paper from the Department of Genetics, College of Agriculture, University of Wisconsin, No. 359.

not persist in the mature seed, or occurs therein as a remnant only. Sometimes, as in the Gramineae, the endosperm becomes a massive storage organ; but this is a secondary use superadded to the primary one.

The problems which the endosperm presents stem from the peculiarities of its origin and genetic endowment and its intercalary position between the old and the new sporophytes. It is now known that early seed development is a critical stage in the ontogenetic cycle of flowering plants. This arises primarily from the circumstance that the conditions essential for growth and differentiation of the zygote into an embryo are not present at the time of fertilization, as in the gymnosperms, but are generated as the ovule becomes transformed into a seed. The endosperm is a special means of mediating the necessary developmental changes. If the tissue does not succeed in its function the matter is of some moment because the embryo which is dependent upon it then fails also.

ORIGIN AND DEVELOPMENT

Female Gametophyte

Double fertilization provides the initial stimulus for the metamorphosis of the angiosperm ovule into a seed. Each ovule is borne at the apex of a funiculus or stalk which arises from the placenta, a portion of the inner surface of the ovary.

The ovule consists of a central portion, the megasporangium or nucellus, surrounded by one or two integuments with an opening, the micropyle, opposite the apex of the nucellus. A subepidermal cell of the nucellus immediately opposite the micropyle, either at once or after division, enlarges and differentiates as a megasporocyte. This diploid cell increases in volume until it becomes several times its original size, and then, in the majority of species thus far examined, by two successive divisions (meiosis) gives rise to an axial row of four haploid megaspores. The outer cell may not divide again after the first division, so that the quartette is incomplete. Usually the innermost megaspore develops into the female gametophyte, and the remaining spores disintegrate. This functional spore enlarges and elongates, and as a result of a series of divisions of its nucleus eight nuclei are formed which are arranged in two groups of four, one at each end of the developing gametophyte. During the last mitoses, cell plates are formed across

the spindles in such a manner as to cut off three cells at each end of the gametophyte and leave one nucleus from each group in the central cell, so that a seven-celled, eight-nucleate structure is formed. The three cells at its micropylar end become differentiated into an egg and two synergids (the egg apparatus), while those at the opposite end (the antipodals) remain more or less alike. The two free nuclei (the polar nuclei) in the large central cell come to lie side by side and may even fuse prior to maturity of the gametophyte. The two synergid nuclei are sister nuclei, *i.e.*, they arise after division of a single nucleus. The egg nucleus and upper polar nucleus are likewise sister nuclei. This type of monosporic gametophyte development is known as the "normal type". A tabulation, based on Schnarf's (217) extensive collation of cases, reveals that exceptions to the normal type of female gametophyte development have been reported in only 13% of the angiosperm genera. Extensive summaries of the various types of development of the female gametophyte are to be found in other papers (154, 155, 219).

One other type of female gametophyte arises from a single megaspore, the so-called *Oenothera* type which is limited to the family Onagraceae. The gametophyte usually develops from the micropylar spore. As a result of two mitoses followed by cytokinesis, a four-celled female gametophyte is produced consisting of two synergids, an egg and a large uninucleate chalazal cell.

A bisporic gametophyte may develop from one of the two cells formed following meiotic division I. Two of the nuclei resulting from meiotic division II undergo two further divisions to yield an eight-nucleate, seven-celled gametophyte (*Allium* type). In some genera meiosis is not accompanied by cytokinesis, and the four spore nuclei enter into the formation of a tetrasporic gametophyte. All four nuclei may divide once again to form an eight-nucleate gametophyte (*Adoxa* type), or they may divide twice giving a 16-nucleate gametophyte (*Peperomia* type). The number of polar nuclei in the mature gametophytes following the latter type of development varies from two to 14.

Another variation of the tetrasporic gametophyte occurs following the *Fritillaria* type of development. No cytokinesis follows meiosis, and one of the spore nuclei remains at the micropylar end of the cell, while the other three pass to the chalazal end. All then undergo mitosis. Two haploid nuclei are formed at the micropylar

end. The three nuclei at the chalazal end undergo fusion during mitosis so that two triploid nuclei are formed. The four nuclei now in the gametophyte, two haploid and two triploid, undergo another mitosis. The synergids, egg and upper polar nucleus of the mature gametophyte are haploid, whereas the lower polar nucleus and three antipodal cells are triploid. Reduced types of tetrasporic gametophytes are common in the Plumbaginaceae (*Plumbago* and *Plumbagella* types).

Thus meiosis and nuclear fusion result in qualitative differences among the nuclei in certain female gametophytes. The number of polar nuclei and the quality of their gene complexes, following the different types of development, vary widely. These phenomena are infrequent, but their significance is obvious to the geneticist. The female gametophytes in the angiosperms are extremely reduced when compared with those of the other group of seed plants, namely, the gymnosperms.

Origin of the Endosperm

After pollination the pollen germinates on the stigma and puts forth a pollen tube which makes its way down the style into the ovarian cavity and along either the ovary wall or the placenta to the insertion region of the ovule. The two male gametes containing sister nuclei are carried to their destination in the tip of the pollen tube which continues along the surface of the ovule to the micropyle. The pollen tube enters and passes through the micropyle to the apex of the megasporangium. It then penetrates the nucellus and advances into the female gametophyte between the cells of the egg apparatus where its tip enlarges somewhat and the gamete nuclei are discharged. One male gamete nucleus enters the egg cell and the two nuclei conjugate forming the zygote. The second male gamete nucleus enters the central cell and unites with the fusing polar nuclei to form a primary endosperm nucleus. The conjugation of one male nucleus with the egg nucleus in the formation of the zygote, and participation of the second male nucleus in the triple fusion to form the primary endosperm nucleus is a process peculiar to the angiosperms and has been termed "double fertilization". It was first described by Nawaschin (185) and Guignard (86).

The zygote gives rise to the embryo. The primary endosperm

with a triple fusion nucleus, by repeated divisions, forms the endosperm. This is usually a short-lived nurse tissue. Participation of a male nucleus in the triple fusion has given rise to much discussion regarding the morphological nature of the resulting endosperm tissue, and until recently little attention has been given to the physiological significance of this event.

The Endosperm in Angiosperms and Gymnosperms

The older view (46) was that, since the endosperm of angiosperms is a nutritive tissue at whose expense the embryo grows, it should be considered as a gametophytic tissue. This view arose in an attempt to homologize this tissue with the so-called endosperm of the gymnosperms which is derived by continuous cell division of the haploid megaspore and is obviously gametophytic. The endosperm in both groups is a relatively undifferentiated tissue and its rôle is essentially nutritive. A brief review of the salient features of the development of the endosperm in the gymnosperms will show that it is not comparable to that in the angiosperms.

The extensive mass of undifferentiated tissue of the female gametophyte in the more primitive gymnosperms, the cycads, for example, is packed with storage materials and constitutes a large nutritive reserve which is available to the zygote. Abundant food for the development of the young embryo is present, therefore, at the time of fertilization. A progressive simplification of the female gametophyte occurs as one passes toward the higher gymnosperms, and there is a tendency for an earlier differentiation of the egg in the ontogeny of the gametophyte. *Gnetum gnemon* stands at the upper extreme in this respect, since the gametophyte is a single multinucleate cell at the time of fertilization and any one of its nuclei is a potential egg nucleus (44).

This cell is relatively low in reserve nutritive materials, and the compact nucellar tissue at the chalazal end of the gametophyte, the so-called "pavement tissue", serves as a primary source of food in the early development of the endosperm. Even in the higher gymnosperms there is nothing to suggest a fusion of a second male gamete nucleus with one or more nuclei of the megagametophyte, such as characterizes the angiosperm endosperm. Coulter, Barnes and Cowles (47) suggest that much of the confusion concerning the morphological nature of the endosperms in angiosperms would

be eliminated if the term "endosperm" were limited to this group, and that the so-called "endosperm" in the gymnosperms be designated as "female gametophyte".

Concurrent with the simplification of the female gametophyte in the gymnosperms there is an increasing persistence of the nucellus. This tissue in the more primitive forms, such as *Cycas* and *Ginkgo*, is almost completely obliterated in the course of development of the gametophyte (30, 48), whereas in the higher forms, such as *Torreya* (49) and *Gnetum* (44, 73), it is still present and functioning at the time of fertilization. In *Torreya taxifolia* both gametophyte and endosperm are stimulated to active development, growth of the one tissue being in inverse relation to that of the other, indicating that a competition exists between the two tissues for available food materials. The gametophyte expands at the tip of the nucellus where it is inactive and obliterates all but a few peripheral layers of cells. The more actively growing nucellus below the apical region, the so-called perisperm, resists the invasion of the expanding gametophyte, especially opposite the vascular strands, so that its surface is very irregular. Both gametophyte and perisperm persist as storage tissues in the mature seed. In *Gnetum*, on the other hand, the proembryos grow over the surface of the generative portion of the gametophyte which is gradually destroyed until only a cavity remains. The vegetative portion of the gametophyte and the nucellus expand rapidly and persist as storage tissues.

Some workers (30, 48) have emphasized that the endosperm is characteristically an aggressive tissue, even in such forms as *Torreya* where its expansion is resisted by the growing perisperm. Others (19) suggest that the margin in relative aggressiveness which the endosperm must hold over the adjacent parts of the seed in order to insure nourishment of the young embryo appears to approach the effective minimum in the higher gymnosperms.

The angiosperm ovule, in contrast to that of the gymnosperm with its considerable storage of reserve foods, contains little or no reserve foods at the time of fertilization. The female gametophyte, in the typical case possessing only eight nuclei, likewise is reduced in size below that of the most advanced gymnosperms, and no endosperm is formed until after fertilization. As a result of fertilization, the endosperm, embryo and the quiescent cells of the surrounding maternal tissues are stimulated to active growth. Since there is

relatively little reserve food at the time of fertilization and storage products are not laid down until late in the development of the seed, the embryo develops at a nutrient level which provides only for its immediate needs. The female gametophyte is very small relative to the total mass of the ovule, and both endosperm and embryo must compete with the adjacent well established tissues for the limited nutrient supply. Brink and Cooper (19) interpret double fertilization as a mechanism whereby the physiological advantages of hybridity become available to the endosperm, thus assisting it to become the most aggressive portion of the developing seed at the earlier stages of development. They suggest also that the secondary fertilization is a device tending to compensate in the reproductive process for the extreme reduction of the angiosperm female gametophyte.

Numerous theories have been advanced in attempts to homologize the female gametophytes of angiosperms and gymnosperms. Porsch (197, 198) maintained that the eight-nucleate female gametophyte of angiosperms is actually a complex of two archegonia without any prothallial tissue. The micropylar archegonium consists of the two synergids, the egg and the upper polar nucleus, the synergids being equivalent to neck canal cells, and the polar nucleus to the ventral canal cell, while the chalazal but now functionless archegonium consists of the three antipodals and the lower polar nucleus. Land (139) observed that the ventral canal cell of *Thuja* may occasionally divide after fertilization of the egg and may even fuse with a sperm prior to such division. This finding led Porsch to the view that the polar nucleus in angiosperms is homologous with the ventral canal cell of gymnosperms.

Schürhoff (221) did not consider the synergids as sister cells, but rather that the egg and one synergid are sisters and that the other synergid is a sister to the upper polar nucleus. He regarded the one synergid and the egg as constituting a reduced archegonium, the synergid being equivalent to the ventral canal cell and the remainder of the gametophyte being of a prothallial nature, partly cellular (the synergid and antipodals) and partly multinucleate (the binucleate endosperm mother cell). Later (223) he revised his interpretation and concluded that two archegonia are formed, each consisting of a ventral canal cell (synergid) and either an egg or potential egg (upper polar nucleus). The eggs of both arche-

gonia are fertilized, each by one of the male gametes from a single pollen tube. The embryo from the potential egg (the endosperm) becomes used up in the nutrition of the true embryo. This, according to Schürhoff, is but an advanced condition over the type of fertilization found in certain of the Coniferales (48) where, if the contents of the pollen tube are discharged into the archegonial chamber, two eggs are usually fertilized by the two male gametes. Schürhoff believed that his theory explains double fertilization and that the endosperm is, in reality, a second embryo, even though it is later suppressed by the embryo arising from the egg.

Schürhoff's theory rests on the incorrect supposition that the two synergids are not sisters to each other. Further it does not take into account the fusion of the two polar nuclei with the male nucleus in the formation of the primary endosperm nucleus. It is to be emphasized that, wherever definite observations have been made, the two synergids, on the one hand, and the egg cell and upper polar nucleus, on the other, represent sister nuclei (for reviews see 28, 116, 141, 154, 155, 217-219), and no reliable observations are at hand to substantiate any other origin.

Fagerlind (73) makes a comparison between the female gametophytes of *Gnetum*, an advanced gymnosperm, and that of the angiosperms. A multinucleate gametophyte is formed in *Gnetum*. Later peripheral layers of cells are cut off and the gametophyte gradually becomes cellular, as in *G. latifolium funiculare*, or a single outer layer of cells surrounding a large multinucleate central cell is formed, as in *G. gnemon*. Several of the surface cells at the micropylar end in the formed species function as eggs, whereas in the latter a single cell differentiates and becomes the egg. These egg cells are reduced archegonia. The female gametophyte of *G. latifolium funiculare* is thus intermediate between that of a typical gymnosperm and *G. gnemon*.

Fagerlind considers the cell groups in the angiosperm female gametophyte to be homologous to the cellular coat in *G. gnemon*, i.e., the egg cell corresponds to the reduced archegonium and the synergids and antipodals represent the cellular coat. The secondary endosperm formed in the angiosperm is considered to be homologous to the retarded prothallium in *Gnetum*. The failure of nuclear fusion in the formation of the endosperm of *Gnetum*, as compared with its presence following double fertilization in the angiosperms,

and the presence of a $3x$ chromosome number in the endosperm of those plants having a normal type of embryo sac development, make it difficult to homologize the endosperms in the two sections of the spermatophytes.

The Fusing Nuclei

The egg nucleus and the two polar nuclei of a monosporic female gametophyte, since they are produced after divisions of a haploid nucleus, have like chromosome numbers and gene complexes. When the gametophyte is a result of either the bisporic or tetrasporic mode of development these nuclei may vary both genetically (*Allium*, *Peperomia*, *Adoxa* and *Fritillaria* types) and in number of chromosomes (*Peperomia* and *Fritillaria* types).

The two male gametes arise from a haploid spore, and their nuclei are alike in both chromosome number and gene complex. Both male gamete nuclei enter the female gametophyte, one fusing with the egg nucleus in the formation of the zygote ($2x$) and the other uniting with the polar nuclei or their fusion product to produce the primary endosperm nucleus. If the gametophyte is of the normal type, the zygote nucleus ($2x$) and the primary endosperm nucleus ($3x$) are alike qualitatively, except that the latter has two gene complexes of megaspore origin. Following the *Oenothera* type of development, both zygote nucleus and primary endosperm nucleus are alike in all respects. When the gametophytes are either bisporic or tetrasporic in their origin, the gene complexes of the polar nuclei are unlike, and upon fertilization the primary endosperm nucleus differs qualitatively from that of the zygote. The number of genomes present varies according to the type of female gametophyte development. Due to the introduction of the gene complexes of the two male gamete nuclei at the time of double fertilization, the genetic composition of both embryo and endosperm normally varies from that of the adjacent maternal tissue.

Atypical Chromosome Numbers

The number of chromosomes in the nuclei of the endosperm varies with the type of development of the female gametophyte. Only one polar nucleus is formed in the *Oenothera* type of gametophyte, and the fusion of this nucleus with a sperm nucleus produces a $2x$ primary endosperm nucleus. Both embryo and endosperm

were found to be diploid ($2n = 14$), following the cross *Oenothera biennis* ($n = 7$) \times *Oe. muricata* ($n = 7$) (207). The number of chromosomes in the endosperm nuclei of *Lilium Henryi* is 60, that is to say, $5x$ (35). This is due to the fact that the female gametophyte follows the Fritillaria type of development. The sperm nucleus fuses with one haploid and one triploid polar nucleus to form a pentaploid primary endosperm nucleus. Seven or more nuclei fuse to form the endosperm mother nucleus following the Peperomia type of development. This fusion nucleus unites with the sperm nucleus, and the resulting primary endosperm nucleus is thus a high polyploid. In *Peperomia pellucida* (108), *P. reflexa* (75) and *Acalypha indica* (157) dividing endosperm nuclei have been found with large spindles and a great number of chromosomes which were so densely packed on the equatorial plates that it was practically impossible to make accurate counts. Polyspermy may, in some instances, account for the increase in chromosome number, as in *Acacia Baileyana* (187).

Heterofertilization

Usually the two male gametes from a single pollen tube enter into the process of double fertilization, and the embryo and endosperm are genically identical. Occasionally more than one pollen tube may enter a female gametophyte (217), and more than two sperms are discharged therein. Friesendahl (78) observed a female gametophyte of *Myricaria germanica* containing six male gamete nuclei, and Håkansson (93) found three sperm nuclei within a single egg of *Levisticum*. In such instances only one sperm nucleus conjugates with the egg nucleus, another with the two polar nuclei and the remaining male nuclei disintegrate. The two male gametes which participate in double fertilization may, in such instances, be from different pollen tubes, in which case the embryo and endosperm are unlike genically.

In making a study of the inheritance of scutellum color in maize, Sprague (231, 232) found instances of an aberrant condition in which the endosperm and embryo were of different phenotypes. Normally kernels with white aleurone have no purple or red scutellum color. Many aberrant kernels were found which possessed white aleurone, and the accompanying embryo had a colored scutellum. Since the progeny obtained from such kernels segre-

gated for aleurone color in ratios characteristic of those obtained only from hybrid kernels with colored aleurone, he concluded that the egg and the polar nuclei had fused with sperms of unlike genotypes, and called the process by which this is accomplished "heterofertilization".

PHYSIOLOGICAL MORPHOLOGY

Types of Endosperm

Three structural types of endosperm may be distinguished when one considers the mode of origin, namely nuclear, cellular and helobial (217, 218). Regardless of the mode of origin all types ultimately become, for the most part, cellular.

1. Nuclear type. The nuclear type of endosperm develops from the fertilized central cell of the female gametophyte, the so-called primary endosperm cell, which is usually associated with a large many-celled megasporangium. Its nucleus divides shortly after fertilization and undergoes a series of mitoses during which time the endosperm initial becomes a large multinucleate cell. The nuclei are arranged in the thin layer of cytoplasm at the periphery of the cell, and the central region is occupied by a large vacuole. The layer of cytoplasm is thicker and denser in the chalazal region and adjacent to the embryo, and there is a greater accumulation of nuclei in these regions than elsewhere. Nuclear division is rapid, and the endosperm expands at the expense of the nucellus. This expansion continues until the greater portion of the nucellus is digested and absorbed, the rate of increase in size diminishing as the nucellus approaches exhaustion.

Cytokinesis is then initiated, and the endosperm usually becomes entirely cellular. Such cell division may originate at the periphery of the endosperm and advance toward its center, or at the micropylar end and progress toward the chalaza. When cell formation is initiated at the micropylar end it may advance until the endosperm is completely cellular, as in *Silene* (210), or it may cease at an earlier stage of development so that a chalazal cap of multinucleate endosperm is present at later stages of seed development, as in *Melandrium* (210). An intermediate type of development, wherein the last cells to be formed are multinucleate producing a "basal apparatus", has been described by Schürhoff (223).

Cytokinesis may also advance chalazalward along the periphery

of the endosperm so that the central vacuole becomes surrounded by a sheath of cells. Once this stage is reached the mitotic spindles are at an angle to the long axis of the endosperm, and this central region becomes filled with cells, as in *Zea mays* (202). In some species this peripheral endosperm becomes only a few layers of cells in thickness and a central non-cellular region persists throughout the development of the seed (*Juglans regia*) (184).

The larger portion of the endosperm is consumed while it is in the free-nucleate condition in some species, e.g., *Mirabilis Jalapa* (282), *Alternanthera sessilis* (117) and *Boerhaavia diffusa* (122). Cytokinesis is limited to the micropylar region, and a cap of endosperm cells is formed about the apex of the radicle.

Regardless of the type of cell formation, the peripheral portion of the endosperm directly opposite the tip of the vascular bundle, which enters the growing seed through the funiculus, becomes differentiated into an absorbing tissue. Thereafter the nutritive materials required for further growth of the endosperm and of the associated embryo, for the most part enter the endosperm through this absorbing tissue. The stage of development at which cytokinesis is initiated varies among the many species of plants having the nuclear type. The free-nucleate stage may be of short duration, cytokinesis taking place very early in endosperm development, as in *Asclepias cornuti* (80) and *Hamamelis virginiana* (225). On the other hand, the endosperm may persist as a free-nucleate structure for several days or even weeks, and then become cellular within a very short period of time. The number of free nuclei in such an endosperm at the onset of cytokinesis is very large, ranging from a hundred or more in *Juglans regia* (184) and *Zea mays* (202) into the thousands in *Asparagus officinalis* (208), *Tilia platyphyllos* and *Malva palmata* (238).

2. Cellular type. Nuclear divisions are followed by cell divisions throughout the entire course of development of the cellular type of endosperm. The female gametophytes of species possessing the cellular type of endosperm grow at the expense of a much reduced nucellus. By the time the gametophyte has reached maturity the cells of the nucellus have, for the most part, collapsed and disintegrated, and only a few turgid nucellar cells remain. These are located at the base of the megasporangium between the chalazal end of the gametophyte and the undifferentiated cells of the funiculus

at the apex of the conducting tissue. Following the stimulation for renewed growth provided by fertilization a cellular endosperm is formed. During the course of its development the peripheral cells opposite the chalazal pocket become densely cytoplasmic and act as absorbing cells, as in *Lycopersicon pimpinellifolium* (41).

During an early stage of its development a few cells at the chalazal end of a cellular endosperm, and in some species at its micropylar end as well, may become differentiated as elongate haustorial cells. Those at the chalazal end push between the collapsing basal nucellar cells and come into direct contact with the undifferentiated cells of the funiculus, the so-called chalazal pocket. These haustorial cells form a direct bridge between the conducting tissue in the funiculus and the rapidly expanding endosperm for the translocation of nutritive materials. They may remain uninucleate, as in *Empetrum nigrum* (211), certain members of the Crassulaceae and Bignoniaceae (160, 161, 164, 165) and in *Phryma leptostachya* (36), or they may become multinucleate, as in *Stachys silvatica* (216).

Variations of the cellular type of endosperm formation occur in some species. For instance, the primary endosperm cell of *Phacelia Parryi* divides twice to give a linear row of four cells (244). Each of these then becomes multinucleate and later cellular. Certain cells are differentiated as haustoria very early in the development of the endosperm of *Nemophila insignis* (244). The primary endosperm cell divides transversely to form two cells of approximately equal size. The micropylar cell then undergoes a second division so that a linear row of three cells is formed. The bulk of the endosperm is then produced as a result of divisions of the middle cell. The chalazal and micropylar cells do not divide further but increase greatly in size forming large uninucleate haustoria. A somewhat similar course of development is found in many of the Labiatae (121), except that the haustorial cells become multinucleate.

3. Helobial type. The helobial type of endosperm development, so designated because of its wide occurrence in the Helobiae, is intermediate between the nuclear and cellular types. That it is an intermediate type is attested by the fact that it is seldom found to be characteristic of all members of a single family (217, 218), even though it is widely scattered among the angiosperms. The primary endosperm cell, following the first division of its nucleus, becomes

divided by a transverse cell plate into two cells of unequal size, the micropylar cell usually being several times larger than the chalazal cell. The nucleus of the micropylar cell then undergoes a series of mitoses and the cell expands so that a large multinucleate cell is formed. Later, cytokinesis is initiated, as in endosperms of the nuclear type, and the micropylar portion of the endosperm becomes cellular.

The chalazal cell may undergo no further divisions and remain uninucleate, as in many members of the Crassulaceae where it increases greatly in size and acts as a haustorium (161). Its nucleus may, on the other hand, undergo one (*Sagittaria sagittifolia*) (55) or a short series of mitoses (*Hypoxis decumbens*) (238) to form a multinucleate cell. This large haustorial cell persists during the course of seed development. The chalazal cell in some instances divides a varying number of times to produce a small cellular endosperm between the apex of the funiculus and the main portion of the endosperm, as in *Mitella diphylla* (54).

A deviation from the helobial type of endosperm development, known as the Lycopsis type, was first described for *Lycopsis arvensis* (243). The primary endosperm nucleus undergoes a series of two divisions to form four free nuclei, after which a wall forms segregating two of the nuclei into a small lateral cell rather than into a chalazal cell. These nuclei undergo one further mitosis, after which cytokinesis occurs and four uninucleate cells are formed. These cells increase greatly in size and act as haustoria. The two nuclei in the large micropylar cell undergo a series of mitoses, and a large number of free nuclei are formed prior to cytokinesis. A similar type of endosperm development occurs in *Nonnea*, *Pulmonaria* and *Symphytum* (52).

4. Unclassified examples. The type of endosperm development in some species cannot be assigned to any of the foregoing classes. The primary endosperm cell in *Impatiens Roylei*, for instance, divides unequally to form a small micropylar and a large chalazal cell (56). The former, as a result of two successive divisions, divides to form a linear row of three small cells during which time a series of mitoses is occurring in the latter to produce a multinucleate condition. The apical cell develops into a giant haustorium which extends through the micropyle, sends branches into the funiculus and acts as an absorbing organ. The second cell forms a cellular endo-

sperm immediately surrounding the embryo. The third cell becomes multinucleate and ultimately fuses with the chalazal cell. Later, cytokinesis occurs so that the endosperm becomes, for the most part, cellular.

A somewhat similar deviation occurs in certain members of the Acanthaceae (162). A three-celled endosperm is formed as a result of two transverse divisions of the primary endosperm cell. The central cell, which is the largest of the three, expands rapidly, and after a series of mitoses becomes multinucleate. Later, cell division is initiated in the region of the embryo and advances toward the chalaza until that portion of the endosperm derived from the central cell is entirely cellular. Only one or two mitoses occur in the chalazal cells and they remain in a binucleate or tetranucleate condition. The chalazal cell expands to form a large absorbing organ between the central portion of the endosperm and the apex of the vascular bundle. The micropylar cell expands, grows out through the micropyle and enters the funiculus where its tip branches, each branch advancing to the vascular tissue. These cells act as auxiliary absorbing organs and transfer the nutritive materials from the conducting tissue to the rapidly expanding central portion of the endosperm. Nuclear divisions may occur during the development of these micropylar haustoria so as to form binucleate structures, as in *Ruellia* sp., *Hemigraphis primulaefolia*, and others, or multinucleate structures, as in *Beloperone angustifolia* (162).

Time Relations

Fertilization of the egg and central cell are parallel events. The entire ovule is stimulated to rapid growth as a result of this act. The primary endosperm nucleus divides shortly after fertilization, and a series of divisions in rapid sequence occurs, the endosperm becoming an actively growing tissue within a short period of time. The zygote in some species divides concurrently with the primary endosperm nucleus so that a two-celled proembryo is associated with a two-nucleate endosperm, as in certain of the Alismataceae (53), or with a two-celled endosperm, as in *Plumbago zeylanica* (51), *Valeriana officinalis* (7) and *Vallisneria spiralis* (156). The rate of mitosis in the endosperm in such instances exceeds that in the embryo so that a sparsely celled embryo becomes associated with a greatly expanded endosperm. Usually, however, a period

of maturation of the zygote occurs prior to its division during which time active mitosis is occurring in the endosperm. The extent to which the endosperm develops prior to the division of the zygote varies widely. Whereas the endosperm may contain relatively few nuclei (8-16) in some species, viz.: *Triticum vulgare* (15) and *Medicago sativa* (19), at the time of division of the zygote, in others it may contain a thousand or more, as in *Primula officinalis* (51), or may practically complete its growth prior to such division, as in *Fouquieria* (107).

There are a few sexual species in which it has been reported that the division of the zygote precedes that of the primary endosperm nucleus, namely, *Najas major* (87), *Limnocharis emarginata* (96), *Elodea canadensis* (283), *Limnophyton obtusifolium* (183) and *Vallisneria spiralis* (205). Since in the last two mentioned species such observations have been proven later to be erroneous (110, 156, 158), it is suggested that the early stages of seed formation in the others merit reinvestigation.

The extent to which the endosperm develops varies widely among the angiosperms. It may be completely or almost completely suppressed, as in the Podostemaceae and Orchidaceae, it may be used up in the course of the development of the seed or it may develop into a massive tissue which persists as a storage organ in the mature seed. In the Orchidaceae the basal cells of the proembryo develop into haustoria which extend through the micropyle into the funiculus and may even penetrate the placenta. These cells act as absorbing cells and conduct nutritive materials to the growing embryo.

Usually the endosperm expands rapidly during the early stages of seed development and then is digested and absorbed during the growth of the embryo. The rate of expansion gradually slows down as the nucellus and nutritive layers of the integuments approach exhaustion, and thereafter growth is much slower. The early development of the embryo prior to differentiation of the cotyledons is very slow, and thereafter growth is rapid. The cotyledons digest their way through the endosperm. The cells of the endosperm adjacent to the embryo break down and disappear until little if any of the endosperm remains in the mature seed. During the later stages of development the cells of the cotyledons become packed with nutritive materials. Such endosperm as remains consists of crushed fragments and thin papery remnants.

The endosperm is not completely consumed by the growing embryo in some species, but persists as a storage tissue in the mature seed, as in the cereals, date, coconut, buckwheat, pepper and others. The stored nutritive materials are then available to the young seedling. The cotyledons, and in some species the embryo, are poorly developed in the mature seed, and there is little if any nutritive material stored in any portion of the embryo.

The Perisperm

The storage organ in the seeds of some species is not the endosperm but the perisperm which is of nucellar origin. The central portion of the nucellus remains intact and expands somewhat during early development of the seed. Later this tissue becomes packed with nutritive materials which are consumed by the embryo during germination, as in certain of the Centrospermae (36, 117, 118, 122, 163, 210).

The central portion of the nucellus may, on the other hand, be consumed by the expanding endosperm, as in *Cleome Chelidonii* (200), leaving a few peripheral layers of cells intact. The endosperm is completely absorbed by the developing embryo so that the mature embryo is in direct contact with the nucellar coat. The cells of this nucellar tissue undergo slight modification at the later stages of seed development, become packed with nutritive materials and serve as an auxiliary storehouse in the mature seed. Such storage tissue has been described for various members of the Capparidaceae (85, 164).

Both the endosperm and perisperm persist as storage tissues in the seeds of some plants. The chalazal end of the endosperm may be embedded in the perisperm, as in *Saururus cernuus* (109), or the endosperm may be almost completely surrounded by this tissue, as in *Canna humilis* and *Brachychilus Horsfieldii* (166). In such instances the endosperm functions as an absorbing organ during germination, obtaining nutritive materials from the perisperm and passing them on to the growing embryo.

FAILURE OF THE SEED DURING DEVELOPMENT

Varying the genetic composition of the endosperm in known ways by controlling the kind of sperm participating in fertilization has proved to be a fruitful means of investigating the properties of the

endosperm and of determining the relationship of this tissue to the other parts of the growing seed. Crosses between species frequently result in seeds which collapse entirely or are shrivelled at maturity and of low viability. Impairment may be induced by still other genetic means. The present writers (19), for example, have shown that the low fertility observed after enforced self-pollination of *Medicago sativa* is due not only to a low rate of fertilization but also to frequent breakdown during development of the inbred seeds. This species is partially self-incompatible and hence is normally cross-pollinated. Seed abortion is likewise recognized as a major factor in the sterility accompanying crosses between diploids and their respective autotetraploids (41, 212).

The study of seed development following wide crossing is of especial interest for the general problem of hybrid incompatibility. A variety of physiological mechanisms are effective in preserving the integrity of species. Endosperm disfunction following interspecific hybridization appears to be an important one in the angiosperms. There are other isolating mechanisms operating during reproduction, however, which should be carefully distinguished from it. Interspecific hybridization may be blocked, for example, by failure of the pollen tube to develop to the extent necessary for fertilization. The interchange of genes between two species is also prevented if growth of the hybrid is arrested at the seedling or a later vegetative stage. Still other forms of incompatibility manifest themselves at sporogenesis in hybrids and in the development of male and female gametophytes. Obviously hybrid incompatibility, which has been applied to all these various barriers to effective interspecific hybridization, is a collective expression. It has a certain usefulness in referring to genetic unconformity in general, but the term covers such a diversity of phenomena as to have little descriptive value in a developmental sense. The various processes which the expression covers possibly have little in common except the same end result, namely, a break in genetic continuity.

Substitution of one male parent for a different one in a mating alters the genetic composition of both endosperm and embryo, as a consequence of double fertilization. Attempts have been made to account for seed collapse in terms of embryo disfunction as the primary factor. Even when not expressly stated, the tacit assumption is frequently made that seed failure is fundamentally a matter

of embryo lethality. This appears to be a carry-over of the point of view prevailing in studies on comparative morphology of the developing seed in which the interest has focussed rather sharply on the embryo rather than on the seed as an ontogenetic unit comprising several interdependent parts. There is now at hand considerable genetic and physiological evidence, however, which is not reconcilable with this hitherto prevalent interpretation. The retarded growth or death of the embryo frequently appears not to be a function of the embryo itself, but is a secondary phenomenon originating in abnormal development of other parts of the seed, notably the endosperm. The evidence bearing on this problem is brought together below, according to the several families in which such studies have been made.

GRAMINEAE

Preeminence of the cereals as food plants and the high value of many grasses as forage have stimulated numerous investigations on interspecific hybridization in the Gramineae. The material is well suited also to the study of certain cytogenetic problems. Persistence of the endosperm as a storage tissue and the large size of the normal Caryopsis make the impairment in seed development often associated with wide crossing readily observable. A result is that a larger amount of data on seed development following wide crossing is available from this family than from any other group of plants.

Watkins (267) observed that the cross, *Triticum vulgare* ($2n = 42$) ♀ × *T. turgidum* ($2n = 28$) ♂, yields plump though small seeds which germinate well, whereas the reciprocal cross, *T. turgidum* ♀ × *T. vulgare* ♂, gives shrunken seeds of low germinability. The embryos resulting from these two matings are presumably alike genetically, and Watkins found that the plants to which they give rise were, in fact, indistinguishable. The endosperms from the reciprocal crosses, on the other hand, differ in chromosomal organization. The first mating gives a primary endosperm nucleus containing 56 chromosomes of which 42 are of *vulgare* origin. The second cross yields a primary endosperm nucleus with 49 chromosomes of which 21 are derived from the *vulgare* parent. Assuming that 14 of the chromosomes in the haploid *vulgare* complement are counterparts of the 14 chromosomes in the *turgidum* genom, the

seven chromosomes which distinguish the species are represented in duplicate in the *vulgare* ♀ × *turgidum* ♂ endosperms and only once in *turgidum* ♀ × *vulgare* ♂ endosperms. Watkins suggested that this chromosome difference in the endosperm was responsible for the dissimilarity in plumpness and viability of the two classes of seeds.

Thompson and Cameron (250), in the course of a study aimed primarily toward a determination of the reasons why derivatives with chromosome numbers intermediate between those of the parent species are relatively infrequent among the descendants of hybrids between hexaploid and tetraploid wheats, found clear evidence for selective elimination of certain classes of plants at the seed and seedling stages as a result of inferior endosperm development. The plump seeds formed directly from the mating *T. vulgare* ($2n = 42$) ♀ × *T. durum* ($2n = 28$) ♂ germinated 84% whereas the shrunkened ones from the reciprocal cross gave only 56% germination. These investigators established the further significant fact that individuals arising from wrinkled seeds formed on backcrossing F_1 hybrids to the parental species differed in the inheritance carried from those grown from plump seeds. The results of the mating (*vulgare* × *durum*) F_1 ♀ × *vulgare* ♂ may be cited as an example. Fourteen chromosomes from the *durum* parent conjugate with 14 of *vulgare* origin at meiosis in the hybrid, leaving seven *vulgare* univalents. Since the latter fail to divide at the second meiotic division and move at random to either pole, spores are formed varying in chromosome number from 14 to 21, depending upon the number of univalents received. About one-half the seeds formed from the backcross were classified as "large wrinkled" which, as a group, germinated poorly. Cytological examination of 11 individuals which were reared showed that the eggs from which these plants arose carried 1.8 univalent chromosomes, on the average. Approximately one-eighth of the seeds from the backcross were classified as "large plump". This group, which germinated well, was found to be derived from eggs which had received an average of 3.8 univalent chromosomes. Thompson and Cameron drew the general conclusion that "The endosperm is likely to be poorly developed (and the seeds shrivelled) unless its extra seven *vulgare* chromosomes are completely absent, or completely diploid, or completely triploid. The further it departs from these conditions the greater is the shrivelling. Reciprocal crosses may, therefore, give very different

results, and wide crosses may be possible in only one direction. Unless special care is taken of the shrivelled seeds in species-crosses a fair sample is not obtained in F_2 , since the shrivelled ones contain chromosome combinations not likely to be found in plump ones".

Thompson (247) confirmed and extended these findings in an analysis of the backcross progeny of hybrids between the hexaploid *T. vulgare* and three tetraploid species, *T. durum*, *T. dicoccum* and *T. persicum*. Since the number of chromosomes transmitted by the recurrent parent in the backcross is constant, the variable number contributed by the hybrid may be computed from the chromosome number found in the offspring. Thompson was thus enabled to correlate plumpness and germinability of the seed with chromosome content of the endosperm. A considerable measure of regularity in the results from the different backcrosses was observed, although some exceptions occurred. In backcrosses of the hybrids to the *vulgare* parent, using the latter as the pistillate parent, the seeds were large and plump if the male gametes contained 21 chromosomes or slightly fewer. They were small and plump if the sperm contained 14 or slightly more chromosomes. The seeds resulting from the functioning of sperm with intermediate chromosome numbers were shrivelled. A different class of sperm yielded large plump seeds, namely, those with 14, or 14 plus one or two, chromosomes, if the pistillate parent in the backcross was a tetraploid species. Male gametes with 21 chromosomes, or a number closely approaching this value, gave large wrinkled seeds.

When *vulgare* pollen was applied to the hybrids, most of the eggs and polar nuclei from which plump seeds developed contained 21 or slightly fewer chromosomes. Pollen from the three tetraploid species, when applied to the hybrids, usually yielded large plump seeds if the eggs and polar nuclei involved contained 14 or 14 plus one or two chromosomes.

These results were interpreted by Thompson in terms of the content of the endosperm nuclei with reference to the seven *vulgare* chromosomes which behave as univalents at meiosis in the hybrids. The seeds from the backcrosses are plump and large when the endosperm nuclei contain three complete or nearly complete sets of these chromosomes, or none or few of them. Two sets, complete or nearly so, usually yield small but plump seeds. Haploidy for all or many of the seven *vulgare* chromosomes in question, or diploidy or triploidy for some only, give shrunken seeds.

Thompson (248) saw in these relationships a possible explanation for a significant fact which he emphasized, namely, that in crosses between species different in chromosome number the mating is more likely to yield germinable seed if the parent with the larger chromosome number is used as the female parent. Use of the species with the larger number as the pistillate parent yields a hybrid endosperm in which the chromosomes possessed by this species and absent in the other are duplicated, whereas in the reciprocal cross these chromosomes will be single. The results from numerous reciprocal interspecific crosses in *Triticum* and related genera, *Nicotiana* and *Brassica*, were shown to conform to the rule. Thompson's explanation applies, of course, only in those cases in which fertilization occurs following both of a pair of reciprocal matings. Ability of the pollen tube to reach the embryo sac and thus permit fertilization may differ in reciprocal crosses, as has been found in matings between diploid and tetraploid strains of *Datura* (27) and *Zea* (201). Non-fertilization and very early post-fertilization collapse of the seed are not certainly distinguishable except by histological examination.

Irregular Mitosis in the Endosperm

Kihara and Nishiyama (130) found that *Avena strigosa* ($n = 7$), on being pollinated with *A. fatua*, *A. sativa* and *A. sterilis*, species with $n = 21$ chromosomes, sets seed freely, but the kernels are much shrivelled at maturity and fail to germinate. The respective reciprocal crosses yield a few poorly developed seeds which are occasionally viable. *A. strigosa* when pollinated with *A. barbata* ($n = 14$) or *A. abyssinica* ($n = 14$) likewise forms seeds in a high proportion of the cases, but the kernels are shrunk and do not germinate. Seedlings are obtainable, however, from the smaller number of better developed seeds yielded by the respective reciprocal crosses. F_1 kernels from crosses between 14-chromosome and 21-chromosome *Avena* species are relatively plump and germinate well either way the matings are made.

Early growth of the seeds from a mating of *A. strigosa* ♀ × *A. fatua* ♂ and its reciprocal was compared histologically with that of normal kernels from the parent species (130). Two- to four-celled embryos are found in normally pollinated *A. strigosa* and *A. fatua* seeds at 24 hours. The endosperm elongates toward the chalazal

end of the seed and rapid nuclear division occurs. The endosperm nuclei at this stage lie free in a peripheral layer of cytoplasm. The antipodals are much enlarged, vacuolate to a high degree, and "represent a remarkable hypertrophy". The embryo is many-celled at 48 hours after pollination. The numerous free nuclei in the endosperm are distributed uniformly in a thin sac-like layer of cytoplasm adjacent to the nucellus except in the vicinity of the embryo and opposite the antipodals where the cytoplasm is denser and the nuclei more frequent. The antipodals, which are now lateral to the endosperm as a result of growth of the latter toward the chalaza, are becoming compressed between the endosperm and the nucellus. The antipodal cells are still highly vacuolate and the nuclei markedly hypertrophied. Cell wall formation in the endosperm begins at about 72 hours in the vicinity of the embryo. A layer of small compact cells is first formed on the outside of the endosperm followed by a second layer of larger cells lying anterior to the first. The antipodals have nearly disappeared by this time.

In the *A. strigosa* ($n=7$) ♀ × *A. fatua* ($n=21$) ♂ cross, Kihara and Nishiyama report five- or six-celled embryos at 24 hours. Initially the endosperm grows rapidly, especially near the embryo. Irregularities in the division of the endosperm nuclei are observable at 48 hours. Giant masses of chromatin are formed which in turn may undergo abnormal division. Oversize nuclei in the hybrid endosperm arise in two ways: by formation of restitution nuclei from daughter groups of chromosomes in anaphase or telophase, and by fusion of adjacent dividing nuclei. Nuclear division may proceed apparently normally in some parts of an endosperm which is otherwise highly irregular. The antipodals are said to be "degenerating in the usual way". Many nuclei of variable size, shape and density are scattered about in the endosperm at 72 hours. The cytoplasm has become abnormally vacuolate and cell wall formation is fragmentary. The embryo remains essentially normal in appearance.

Embryo and endosperm development is slower in the reciprocal cross *A. fatua* ♀ × *A. strigosa* ♂ than in *A. fatua* selfed. Evidence of disorganization of the endosperm in the region of the embryo is seen at 48 hours. The tissue is sparsely cytoplasmic and cell wall formation is much restricted. The appearance of the endosperm is variable at 72 hours but growth is generally weak. The embryos

tend to be smaller than those of *A. fatua* selfed and sometimes show symptoms of degeneration.

A novel occurrence in some *A. fatua* \times *A. strigosa* seeds is the formation of "regenerate endosperm" as a compact tissue of normally appearing cells. The relationship of the regenerate endosperm to the antipodals is noteworthy. It is observed that at 48 hours the cytoplasm of the free-nucleate endosperm in the vicinity of the antipodals is significantly denser than that in other regions. Following normal cell wall formation the tissue may proliferate and in some cases eventually displace the original disorganized endosperm. The formation of regenerate endosperm is believed to enable some kernels to develop to maturity and give hybrid plants. In other seeds, however, the embryo may die before the regenerate endosperm develops.

Wakakuwa (266) presents extensive data on seed development in *Triticum* following reciprocal matings involving several diploid, tetraploid and hexaploid species. The percentage of florets setting seed and the germination of the seeds were high in crosses between parents having the same chromosome number. The results of matings between species unlike in chromosome number differed depending on the direction of the cross. The percentage of florets forming seeds was almost normal in low $\text{♀} \times$ high ♂ matings, but the seeds were wrinkled and germinated badly. High $\text{♀} \times$ low ♂ crosses gave poor sets of seed, but the latter were relatively plump and germinated well. The difference between reciprocal matings was most conspicuous in the diploid \times hexaploid combinations.

Wakakuwa made a histological study of seed development up to five days following reciprocal matings between *T. spelta* ($n=21$) and *T. polonicum* ($n=14$), *T. polonicum* and *T. aegilopoides* ($n=7$), and *T. spelta* and *T. aegilopoides*. Quantitative data are not reported, but the rate of endosperm development in the hybrid seeds as measured by the time at which cell wall formation is initiated appears to lag behind that in the seeds of the respective pure species except in the *T. spelta* \times *T. polonicum* and *T. spelta* \times *T. aegilopoides* matings which are equal to *T. spelta* selfed. No mention is made of irregular mitotic behavior in the endosperm. The hybrid embryos throughout were found to be smaller at 120 hours than those of the respective controls. The antipodals enlarge conspicuously during the brief period of free-nucleate endosperm de-

velopment and regress quickly when the tissue becomes cellular. Wakakuwa did not observe any difference in antipodal behavior between the hybrids and controls. Measurements of maximum length and breadth of seed showed that in all cases at 15 hours and 24 hours the hybrids were larger than the selfs. It appears, therefore, that the maternal tissues of the young hybrid seeds expand more rapidly than those of the controls, although endosperm and embryo development is more or less retarded in the former.

The widespread occurrence of mitotic irregularities in the endosperm of *Triticum* following crosses between distantly related species is well established (15). Endosperm and embryo development was found to be regular in *T. vulgare* ($n = 21$) ♀ × *T. durum* ($n = 14$) ♂ seeds but slower than in *T. vulgare* selfed, and the ultimate size attained is smaller. The reciprocal cross gives wrinkled seeds which germinate poorly. Endosperm development subsequent to the latter mating is retarded. The space occupied by the endosperm at 14 days is greater than that in either parent and much greater than that of the reciprocal cross. But cell formation and starch deposition are much slower. Irregularly shaped nuclei were first noticed in the endosperm at three days in the vicinity of the embryo. These are more frequent and widespread at four days. Cell formation is very irregular and incomplete. The present writers have observed that the endosperm, particularly that of weakly developed hybrid seeds, is prone to collapse on fixation, particularly if injured in handling. Some of the abnormal structures which Boyes and Thompson report are probably the result of such injury, but the evidence they present clearly shows that radical mitotic disturbances are a characteristic feature of the endosperm following various *Triticum* species crosses. Early embryo development, on the other hand, is not thus affected.

The Khapli variety of *Triticum dicoccum* ($n = 14$) sets few seeds in crosses with *vulgare* wheats, and the seedlings usually die at an early age. *T. vulgare* ♀ × Khapli ♂ seeds, aside from displaying a somewhat lower rate of endosperm and embryo growth, as compared with *T. vulgare* selfed, and a smaller ultimate size, develop regularly. Following the reciprocal cross the space occupied by the endosperm increases very rapidly, the tissue being three times as long at seven days as that of the reciprocal. Again, however, differentiation is much slower in terms of cell wall formation, de-

velopment of the aleurone layer and starch deposition. Conspicuous and extensive mitotic disturbances are in evidence from four days on.

T. spelta ($n = 21$) ♀ × *T. monococcum* ($n = 7$) ♂ seeds, after a slow start, grow rather well for a period, but endosperm development soon slows down and ceases at seven days. Mitotic irregularities occur but are not frequent. Boyes and Thompson found that endosperm development in the reciprocal cross is extremely abnormal. The volume occupied by the endosperm of the young seed is comparable with that of *T. spelta* selfed, but cell formation is greatly retarded. Small, dense, irregularly shaped nuclei appear, and the cytoplasm may break up into irregular patches. No functional seeds are formed.

Most varieties of common wheat are not fertilized when mated with rye (*Secale cereale*, $n = 7$). The Chinese variety of *Triticum vulgare*, however, gives some viable seeds from pollination by rye. Boyes and Thompson observed that endosperm development in this hybrid is variable. Sometimes it is regular but slower than that in wheat, and in these cases the embryo grows normally. In other Chinese wheat × rye hybrid seeds cell formation in the endosperm is delayed and development may even be arrested. The abnormal nuclear and cytoplasmic conditions observed in certain *Triticum* crosses, however, were not found.

Boyes and Thompson note the conspicuous enlargement of the antipodal cells and nuclei in the normal *Triticum* seed, directly after fertilization, and the recession of this tissue beginning at two days as the endosperm becomes cellular. Data are not presented, however, on antipodal behavior in the hybrid seeds.

Hordeum × *Secale* Seeds

Hordeum jubatum ($n = 14$), squirrel-tail barley, hybridizes readily with common rye when the former is used as the pistillate parent (199, 249). Fertilization likewise occurs regularly when common barley (*H. vulgare*, $n = 7$) is similarly mated with rye. In neither case, however, do germinable seeds develop. The developmental behavior of these *Hordeum* × *Secale* seeds has been studied by the writers (21) and others (251). The findings correspond in several respects but disagree in others, and will be presented separately.

Cooper and Brink (21) report that fertilization occurs in the *Hordeum jubatum* ♀ × *Secale cereale* ♂ mating within four hours after pollination, as in *H. jubatum* selfed. Subsequent development proceeds with remarkable rapidity. The endosperm in the normal seed is sometimes two-nucleate at four hours, and further growth of this tissue continues by synchronous free-nuclear division up to about 32 hours, at which time cell division begins. The tissue consists of a single layer of cells surrounding a central cavity at 48 hours except in the vicinity of the embryo where the cells are several layers in thickness. Endosperm growth is attended by an enlargement of the seed as a whole and a progressive absorption of the nucellus. The lumen of the normal endosperm becomes filled with large, highly vacuolate cells by three days, at which time starch storage begins. The aleurone layer becomes differentiated by four days. The zygote divides to form a two-celled proembryo between 10 and 24 hours. The embryo comprises about 20 cells at 48 hours and 120 cells at 72 hours. The main features of the embryo, as found in the mature seed, are delineated by 10 days.

Division of the primary endosperm nucleus in the *H. jubatum* × *S. cereale* seed is delayed slightly as compared to the control, *H. jubatum* selfed. The number of endosperm nuclei averages less than 10 at 32 hours when the normal endosperm has reached the 128-nucleate stage and is becoming cellular.

Irregular mitotic behavior of the endosperm, which is a conspicuous feature of the relatively short-lived hybrid seed, may appear at division of the primary endosperm nucleus. Incomplete separation of the two daughter groups of chromosomes, leading to dumbbell-shaped interphase nuclei, was observed at this time. Gross mitotic disturbances are the rule from 24 hours on, so that marked diversity in the size, shape, number and position of the endosperm nuclei arises. The chromosomes remain recognizable as distinct entities but the number per nucleus becomes highly variable. No cell walls form in the hybrid endosperm.

The young *H. jubatum* × *S. cereale* embryo develops almost normally, in marked contrast to the more or less disorganized endosperm which accompanies it. Growth, however, is somewhat slower, the hybrid attaining a given cell number about one day later than the control during the period up to 96 hours. Differentiation proceeds normally in the hybrid embryo. The latter,

however, shows progressive signs of starvation as the seed approaches collapse at six to 13 days after pollination.

The fact that the primary endosperm nucleus in the hybrid seed may divide irregularly within an hour or so after fertilization, and the improbability, in view of normal cell division in the accompanying crossbred embryo, that the disturbed mitosis is a direct effect of hybridity of the nucleus, led the writers to explore the changes occurring in the *Hordeum* ovule immediately following advent of the two classes of sperm, *Hordeum* and *Secale*, to the *Hordeum* female gametophyte. Four ovules each from the *H. jubatum* \times *S. cereale* and *H. jubatum* selfed matings were found in the two- and four-hour material at hand in which fertilization had occurred but in which division of the primary nucleus had not yet begun. Three unfertilized ovules collected at four hours after pollination were available for comparison. The volumes of these 11 ovules and of their contained embryo sacs, antipodals and antipodal nuclei were determined from planimeter measurements of outline drawings of the tissues made with a camera lucida from successive 15-micron serial sections. We report the following results (21). Immediately after normal fertilization in *H. jubatum* has occurred and before either the primary endosperm nucleus or zygote divides, the volume of the ovule doubles and that of the embryo sac increases over six-fold. There is an approximately six-fold increase in the volume of the antipodals brought about by the swelling of the approximately 15 cells already present in the mature female gametophyte. The antipodal nuclei also undergo a marked hypertrophy, resulting in an increase in volume of about three times. The response to fertilization by the rye sperm, on the other hand, is much less pronounced. The ovule increases in size very slightly and the embryo sac becomes only about one-half again as large. The antipodals increase in volume about 65%, as compared with over 600% in the normal ovule. There is a correspondingly small increase in size of the antipodal nuclei. It is evident from these facts that nuclear division in the *H. jubatum* \times *S. cereale* hybrid endosperm begins under greatly different circumstances than in normal *H. jubatum*.

A similar series of measurements was carried out on five ovules each of *H. vulgare* selfed and *H. vulgare* \times *S. cereale*. The seeds resulting from the latter cross fail to develop to a germinable condition after undergoing the same gross changes, including abnormal

mitotic division of the endosperm nuclei, as those observed in the *H. jubatum* × *S. cereale* mating. The immediate volume increase, particularly of the antipodals, was less in common barley fertilized by rye than in common barley selfed. In both these matings, therefore, hybrid fertilization is followed by a less pronounced early stimulation of the antipodals and adjacent maternal tissues of the ovule than is the case with normal fertilization.

The highly abnormal course of seed development following pollination of common barley (*H. vulgare*) by rye has been described (251). Embryo development in the hybrid seed parallels that in normally fertilized barley at one and two days. Thereafter the hybrid embryo, although remaining healthy in appearance, lags behind in growth so that at seven days the pure barley embryo is over four times as long and is beginning to differentiate. Little further development of the hybrid embryo occurs, the cells become highly vacuolate and the structure breaks down by 12 days.

Division of the primary endosperm nucleus precedes that of the zygote. A very rapid increase in the number of free endosperm nuclei in the normal barley seed follows. At three days 200–520 nuclei are present, which are distributed throughout the peripheral layer of cytoplasm. Cell walls are formed in the pocket around the embryo at four days, and by five days the endosperm has become a completely cellular layer surrounding a central cavity. The cavity by the sixth day is filled with cells in which starch grains appear.

The primary endosperm nucleus in the barley × rye seed divides as soon as or a little sooner than in barley selfed. Therefore, however, relatively few divisions occur. Rarely are more than 20 endosperm nuclei formed before the hybrid seed collapses. Thompson and Johnston note that, as in several other cereal species crosses mentioned above, the behavior of the nuclei in the common barley × rye endosperm is very abnormal. They point out that even the two nuclei resulting from division of the primary endosperm nucleus may be larger than normal, sometimes very much larger, an observation in agreement with that of the writers on similar material. Some of the endosperm nuclei at two days exceed the size of the entire embryo. Occasional hybrid nuclei were observed eight times the diameter of those in the normal barley tissue. The nuclei varied widely in shape. Three division figures observed were very irregular and disclosed the presence of chromo-

somes in numbers far in excess of the 21 which normally would be present. It is concluded from the size, number and appearance of the hybrid nuclei that the chromosomes may multiply several times without regular mitotic division.

Thompson and Johnston did not observe any difference in antipodal behavior which appeared significant for collapse of the *Hordeum* \times *Secale* seed. Measurement of length of the mass of antipodal cells in selfed and hybrid seeds gave similar values at one and two days and only small differences thereafter. The antipodals in the hybrid seeds, however, persisted somewhat longer. It is concluded that behavior of the antipodals in the crossed material differs only slightly from that of the normal and in ways that would be expected in view of the slower growth of the embryo sac and the collapse of the endosperm.

A detailed examination of the maternal tissues of the caryopsis following the two types of matings disclosed no evidence of overgrowth associated with breakdown of the hybrid endosperm. Previous studies by Thompson on various other wide crosses in the Gramineae involving *Triticum*, *Aegilops*, *Agropyron* and *Secale* had not revealed such an effect. A re-examination of this material by Thompson and Johnston confirmed the earlier observations.

Self-fertilization of Rye

Common rye, *Secale cereale*, develops comparatively few seeds upon selfing as compared to crossing to unrelated individuals. Jost (119) demonstrated that a marked restriction in pollen tube growth was associated with the former mating, and it has been commonly assumed since that this accounted for the self-sterility. Landes (140) has shown, however, that an additional factor is involved, namely, seed abortion. She confirmed the claim that the frequency of fertilization is definitely lower on selfing, but observed also that twice to three times as many young seeds abort after this type of mating as after outcrossing. The actual percentages of seeds collapsing in two successive years were 42 and 82 after selfing as against 24 and 26 for crossing.

Histological study of the rye seeds at five days and later revealed pronounced irregularities in endosperm development. Although some crossed seeds were affected, Landes found that abnormal development was more frequent and, in general, more

pronounced after self-fertilization. Some of the endosperm anomalies which Landes reports are of the kind which the present writers have found to be associated with mechanical injury of the young seed previous to fixation. There are others, however, which are clearly not artifacts. Among the latter are giant, irregularly shaped nuclei, containing seven or more nucleoli, and very dense, small nuclei. Endosperms were observed also in which free nuclei and cellular patches of tissue were irregularly distributed. The general picture recalls the endosperm conditions which have been described for several species crosses in *Avena* (130) and *Triticum* (15), and since for *Hordeum* \times *Secale* hybrids (21, 251).

Defective Seeds in Maize

A heritable recessive condition, termed "defective" seeds, appears rather frequently on self-fertilizing maize (e.g., 111, 148). An extensive analysis of the phenomenon, including observations on the development of defective seeds, has been published by Mangelsdorf (159).

He estimated, on the basis of data from several sources, that one plant in about 30 in open-pollinated varieties of maize is heterozygous for defective seeds. Several different genes producing the character have been identified, and tests indicate that the total number occurring in the species is large. Origin of the character by mutation has been observed in inbred lines.

The extent to which size of the seed is impaired varies greatly in different defectives. Mangelsdorf records one case in which weight of the defective seed on a segregating ear was only 2.4% of that of the normal sister seeds. Mature weight ranged up to 58.8% of normal in other cases. This value for a given defective fluctuates widely, however, depending upon the residual inheritance of the plant on which the defectives occur.

Defective seeds on segregating ears often cannot be distinguished from their normal sibs until 15 days or more after pollination. Since the defectives which Mangelsdorf studied could not be propagated in homozygous condition, he was limited to comparisons between the two classes of seeds at the later stages of development only. A major portion of the total growth of the embryo in normal seeds occurs after 15 days; but the endosperm as a definitive tissue is already far advanced by this time. Beyond 15 days the principal

change in the endosperm is the continuing accumulation of reserve foods.

Mangelsdorf observed that both endosperm and embryo in defective seeds were much retarded in development. The endosperms grew slowly and failed to attain normal size in all cases. An aleurone layer was not formed in the most extreme defectives and was incompletely differentiated in others. The amount of starch deposited in the endosperm cells of the several defectives varied from none to large amounts. In general, there was definite correlation between degree of development of embryo and endosperm, an observation which led Mangelsdorf to conclude that the expression of hereditary factors in the two tissues is parallel. Considerable modification of shape occurred in the embryos of defective seeds, particularly at the later stages of development. Development of the pericarp in the defective maize caryopsis is comparatively little affected. Mangelsdorf noted that the nucellus, which is digested in the normal seed within a few days after fertilization, persists longer in defective seeds. Plants reared from viable defective seeds were weak and spindling throughout their entire growth period.

The present writers have made some preliminary developmental studies on a defective seed type in maize which may be propagated in homozygous condition. This strain was derived from one of several ears segregating for defective seeds obtained by self-pollinating a large number of plants in a yellow dent commercial variety. Dry weight of the mature caryopsis in this defective seed type is only about 25% of that of normal sibs. Germination is poor and the seedlings are very weak and often die. Once the seedling stage is passed, however, the plants grow well. The mature plants are vigorous and fertile although somewhat smaller than sibs from non-defective seeds.

The seedlings from homozygous defective seeds are so weak that few emerge if the seed is sown directly in the field. Strong plants are obtainable, however, by starting the seedlings in the greenhouse and transplanting at about two weeks of age. The homozygous defective individuals shed pollen abundantly and form seed readily. The stock has been propagated for several generations in the homozygous condition.

Growth of the seed of this defective, measured in terms of dry

weight, is equal to that of normal seeds up to nine days after pollination. At 12 days the average weight of normal seeds is slightly greater than that of defectives. The difference, however, is pronounced at 16 days. No further increase in weight of the defective seeds occurs after 24 days, at which time the normal seeds are nearly four times as heavy. The latter class of seeds continues to grow beyond 24 days and reaches a weight at maturity more than seven times that of defective seeds of the same stock.

Histological examination shows that up to eight days there is no detectable difference between defectives and normals in the number of endosperm and embryo cells formed. At 10 days, however, the normal endosperm is larger, although the embryos remain closely alike. The nucellus is less rapidly digested in the defective seeds. The most conspicuous histological difference at this early stage is in the character of the outer layers of endosperm cells opposite the saucer-shaped structure in which the main portion of the vascular bundle terminates. It is through these cells that the nutrients used in growth of endosperm and embryo pass. The outer layers of endosperm cells in this region of the normal seed become densely cytoplasmic and elongated, changes which are indicative of intense activity in absorption. The corresponding portion of the defective endosperm is poorly differentiated. The cells remain rather thinly cytoplasmic and become less elongated in the direction in which the incoming nutrients move.

Starch appears in the endosperm of defective seeds at the same time as in the normal. No difference occurs in the form of the starch grains laid down. The amount of starch stored in the defective endosperms is small from the start, and since growth of these seeds slows down early and ceases at about 24 days, the total reserves accumulated are very meager.

IRIDACEAE

The cross *Iris pseudacorus* \times *I. versicolor* yields shrunken seeds which do not germinate. Sawyer (215) found that selfed seeds appeared to form a few more nuclei in the free-nucleate endosperm than hybrid seeds before 13 days, at which time wall formation was initiated in both cases. Endosperm food reserves were much more abundant in the normal seed than in the hybrid at 20 days. The cells of the hybrid endosperm were almost without contents at

26 days; and at six weeks the tissue had collapsed, forming a dark-colored jacket around the embryo. The endosperm in the normal seed persists to maturity.

Lowig (150) observed that fruits formed after the cross *I. sibirica* ♀ × *I. pseudacorus* ♂ but were significantly smaller than the normal. The ripe capsules contained numerous shrivelled and non-germinable seeds which Lowig believed were without endosperm and embryo. Two possible explanations were suggested to account for these abortive seed-like structures: (a) fertilization had occurred but endosperm and embryo had failed of development, or (b) the empty seeds were the result of a pollination stimulus like that which Fitting (76) had established in the orchid. The reciprocal cross, *I. pseudacorus* ♀ × *I. sibirica*, gave fewer but larger seeds containing identifiable embryos. Lowig was unsuccessful in germinating them.

Crosses between different groups of cultivated irises, according to Werckmeister (272), rarely form normal seeds. Instead of firm endosperm tissue, a pulpy mass develops which becomes brown and hard on drying. Embryos are frequently present but are variously developed. Werckmeister, on observing that sound embryos dissected from mature seeds and placed on a nutrient medium swelled very rapidly and often died, attributed a dual rôle to the endosperm. He believed the tissue provided a nutrient reserve and regulated the absorption of water by the embryo. The seed failure attending *Iris* crosses was mainly due, in Werckmeister's judgment, to lack of development of an endosperm which would function effectively at germination in controlling the rate of water uptake.

URTICACEAE

Humulus lupulus when pollinated by *H. japonicus* forms apparently normal sized fruits, but the embryos are extremely small and are incapable of producing independent plants (253, 278). Tournois (252) crossed *H. lupulus* and *Cannabis sativa* and found that a small embryo was formed in that case also. The *Humulus* species cross was considered by him to involve true fertilization, but he believed that the *Humulus* × *Cannabis* mating led to parthenogenetic development of the *Humulus* egg. The latter conclusion was questioned by Winge who believed that the two cases were alike in principle. *Humulus lupulus* has often been regarded as

occasionally agamospermous. This view is rendered doubtful by the results of the following matings and treatments which Winge carried out:

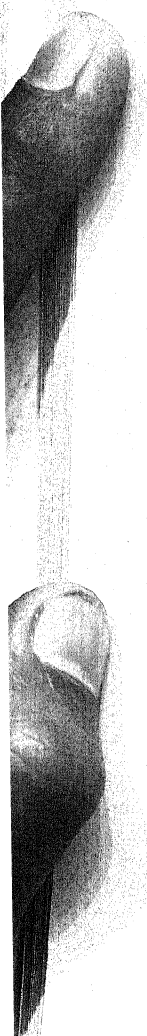
	Mating or treatment	Embryo development
<i>Humulus lupulus</i>	\times <i>Urtica dioica</i>	Fairly good
"	" \times " <i>urens</i>	Extremely strong
"	" \times <i>Cannabis sativa</i>	Good
"	" \times <i>Pilea cynocrambe</i>	Few small embryos?
"	" \times <i>Peperomia resedaeflora</i>	Few small embryos?
"	" etherized	None
"	" isolated under paper bag	None
"	" hop pollen water extract applied	None

The embryos from the *H. lupulus* \times *Urtica dioica* matings were somewhat undersized and the seeds failed to germinate. The fruits were exceptionally well developed, often exceeding in size and weight those of normal hops. Winge suggests that heterogamic fertilization is more often responsible for the occurrence of abortive embryos and parthenocarpic fruit development than is commonly supposed.

ROSACEAE, PARTICULARLY THE POMACEOUS AND STONE FRUITS

The few data available concerning the relation of the endosperm to seed development in the Rosaceae are derived mostly from studies on fruit development in the apple (*Malus sylvestris*), pear (*Pyrus communis*), plum (*Prunus domestica*), peach (*P. Persica*) and cherry (*P. avium*, *P. cerasus*). Although the formation of fruit on these orchard plants varies greatly, depending on nutritional conditions, the process is contingent under ordinary circumstances on the development of seed. It may be expected, therefore, that the endosperm is basically significant for many of the problems in this field, although the direct evidence for this view now at hand is limited and fragmentary.

Only a part, and often only a small part, of the flowers on a normally blossoming fruit tree give rise to mature fruits. Heinicke (102) estimates that it is usually less than 10% in the apple, and Bradbury (16) gives a figure of 25% to 50% for the sour cherry. The remainder are shed, frequently in three rather irregular waves termed first, second, and third or "June" drops, beginning shortly after blossoming starts and terminating several weeks later. The condition of the seeds in dropped fruits has received considerable study.



Müller-Thurgau (172) found that June drops usually contained embryos which had ceased to grow. Kraus (133) emphasized post-fertilization disturbances as a major cause of sterility and unfruitfulness. Dropped fruits in three varieties of apple were found to contain 3.6 seeds, on the average, as compared with 5.7 in those retained on the tree (102). The occurrence of many attached fruits having relatively few seeds and of some drop fruits with a high seed content, however, indicated that other factors also were involved in fruit setting. Vigor of the spur was shown to be an important variable. Heinicke found that "A poorly fertilized flower can develop into a fruit, provided it is borne on a vigorous spur; a weak spur, on the other hand, will mature only a fruit that is developing many good seeds". It was observed also that size of fruit on spurs of a given weight varied with the number of seeds and size of embryos.

Dorsey (65), working with the plum, found that the flowers falling in the first drop possessed aborted pistils, the frequency of which, like fruit bud formation, was correlated with the size of the preceding fruit crop. The remaining pistils in which fertilization had not occurred dropped from two to four weeks after blossoming. The third drop is characterized by fruits of larger size in which fertilization had occurred but in which embryo development had been arrested. Roberts (209) found that practically all second and third drop fruits in the apple contained seeds. He emphasized the nutritional condition of the spur as a factor limiting the number of fruits retained. Thinning of the blossom clusters in the McIntosh and Wealthy varieties increased the setting of fruit from 36% to about 57%. The fruits falling somewhat later than the blossoming stage in the apple, plum, peach, nectarine and sour cherry were found to contain fertile ovules (63). Embryos were present in all but two of 250 third drop cherries (16). Murneek (182) observed that embryos are frequently present in second and later drop apples. There is ample evidence, therefore, that the shedding of young fruits in orchard plants occurs in large numbers after development of the seed begins.

Few data are available concerning the internal condition of the seed in relation to retention and growth of the fruit. There is some evidence, however, that behavior of the seed is significantly affected genetically apart from the medium in which the seed is growing.

Self-incompatibility is common among these plants, and not only is the number of ovules becoming fertile lowered but development of the seed also appears to be adversely affected by enforced self-pollination. Many investigators have found that seed number is regularly higher after cross- than after self-pollination. Alderman (3) notes, for example, that the latter type of mating reduced the seed number one-half to one-sixth in different varieties. In an early investigation on pollination in the pear, Waite (265) reported not only that fruits resulting from selfing contain fewer seeds but also that the seeds are usually abortive. Cross-fertilization, on the other hand, gave rise to fruits well supplied with sound seeds. The writers (20) have suggested, on the basis of observations on *Medicago sativa* seed, that two distinct phenomena are probably involved here. Since the pear is partially self-incompatible, the frequency of ovules becoming fertile is lowered due to restricted pollen tube growth following selfing, and secondly, many fertile ovules fail to develop into mature seeds primarily because the vigor of the endosperm is depressed by the inbreeding. There is some evidence from the orchard fruits themselves that the condition of the endosperm may be a determining factor in seed development.

Bryant (24) has reported that development of the endosperm in the apple is definitely retarded after selfing and that, following compatible outcrossing, the collapse of seeds is reduced in frequency and tends to occur later. It was noted by Bradbury (16) that the seeds in the dropped fruits of cherry have a weakly developed endosperm. Harrold (99), on the other hand, in a study of the aborting and developing fruits of the peach, suggests that degeneration of the nucellus in the chalazal region is the primary factor in collapse. The breakdown in this tissue disarranges the vascular system, and changes in the endosperm and embryo ensue.

Further evidence that the source of the sperm participating in fertilization is important for development of the seed and fruit is afforded by Dorsey's (65) results with the plum. This investigator found that the number of fruits reaching maturity in the Compass variety differed greatly, depending upon whether it was pollinated by the Yellow Egg or the Burbank variety. Of 1327 Compass flowers pollinated by Yellow Egg, 652 fruits began development, but only eight matured. When Burbank was used as the pollen parent on the same pistillate variety, of 175 flowers pollinated, 116

set fruit and 114 matured. Dorsey observes that "A characteristic of all the seeds dissected in the cross Compass \times Yellow Egg was the small amount of endosperm present".

Dorsey (65) considers that the genetic constitution of the embryo in the plum is an important factor in determining whether the associated fruit completes development or falls prematurely. His view concerning the relationship between this factor and the nutrient level of the plant is summarized in the following quotation from his paper. "The influence of unfavorable factor combinations upon the June drop is especially direct in the plum, since there is but a single ovule which develops or not as the case may be. . . . When there is a heavy setting of fruit resulting in increased competition for a relatively limited food supply, the uncongenial factor combinations are the first to cease development, and they are not saved either by a favorable position on the twig or by early fertilization. The difference in the time of falling at the third drop . . . can be explained on the basis that some combinations can proceed farther in development than others".

Early ripening varieties of the sweet cherry (*Prunus avium*), sour cherry and peach form abortive seeds regardless of the source of the sperm participating in fertilization. Varieties which mature at successively later times produce larger, often viable, seeds whose embryos are correspondingly plumper and firmer. Tukey (254, 255) and Tukey and Lee (258) have studied seed and fruit development in these two classes of plants. Three developmental stages between flowering and maturity of the fruit are recognized which Tukey and Lee characterize for the peach in the following way. Stage I extends for about 50 days in all varieties. Rapid growth of the fruit occurs, whereas the embryo remains microscopic in size. During stage II development of the fruit is retarded and the embryo begins a cycle of growth which continues until the maximum size for the variety is attained. The duration of stage II varies directly with the lateness of maturity of the variety. It is only five days long for Greensboro, an early sort, and 42 days for Chili, a late variety. The onset of stage III, varying in time according to the length of stage II, finds the embryos in early varieties still very small, whereas those of late varieties are nearly full size. During stage III, in which the final swell of the fruit occurs, there is an accumulation in the embryo of reserve foods, particularly fats, the amount in the abortive seeds of early varieties being very small.

The formation of abortive embryos in the early varieties of stone fruits is of considerable interest for the study of seed development. Tukey (256) has shown that the collapsing embryos are potentially viable by rearing numerous seedlings from them on artificial media. It is evident, therefore, that the cause of the seed collapse is not to be sought in the embryo itself.

Tukey's results are significant also in showing that death of the embryo in the seed is not due to the premature onset of dormancy, which, conceivably, might be fatal to the immature structure. Excised embryos of a variety which normally develops viable seed were found to germinate readily on an artificial medium 42 days after full bloom, only with difficulty at 56 days, and not at all at 67 days, unless after-ripened under appropriate conditions. The embryos from aborting seeds, on the other hand, could be grown from the time they appeared checked in development, 38 to 43 days after bloom, until 63 days, or until breakdown began. This means that embryos in the class of seeds which aborts never reach the stage of development in which dormancy is involved.

LEGUMINOSAE

The low incidence of seed production following self-pollination of *Medicago sativa* is due to two, probably distinct, factors: partial self-incompatibility resulting in few fertilized ovules, and collapse of seeds during early stages of development (38). About 15% of the ovules in the plants under study were found to become fertile on selfing, as compared with 66% on outcrossing to unrelated individuals. A further fact of particular interest for the present discussion is that, whereas only 7% of the crossbred seeds ceased growth during the first 144 hours after pollination, 34% of the inbred ones collapsed. Brink and Cooper (19) have reported the results of a comparative study of early development of the inbred and crossbred seeds.

Self-pollinations and outcrosses to unrelated individuals were made concurrently on seven plants, using castrated flowers. Pistils were collected for histological examination of the seeds at six periods beginning at 30 hours and concluding at 144 hours. This covers the interval during which the alfalfa endosperm develops as a free-nucleate structure and starts to become cellular and the zygote is transformed into a proembryo and later into a small undifferen-

tiated embryo. The stage of growth in terms of cell number in the embryo and number of nuclei in the endosperm was recorded for about 430 selfed seeds and approximately 1,500 crossed seeds collected over the six-day interval. Observations were made also on the developmental changes occurring in the maternal tissues of the two classes of seeds.

The rate of increase in cell number in the alfalfa embryo during the first six days is low and almost linear. The hybrid embryos, on the average, grow only slightly faster than the inbred ones. At 144 hours, for example, the latter class had a mean cell number of 13.8 as compared with 17.3 for the former. Part of this difference is probably due to the fact that fertilization is delayed somewhat after selfing.

The endosperm, on the other hand, develops with great rapidity. The number of nuclei, lying free in the cytoplasm, increases geometrically by synchronous division. Rate of growth of the hybrid endosperms is significantly higher than that of the inbred endosperms. This is evident both when the comparison is made in terms of number of nuclei present at a given period after pollination and when the numbers associated with a given size of embryo in the two series are examined. The latter measure is preferable in that it is not affected by variations in the time of fertilization. Nine comparisons were possible on this basis, ranging from the one-celled proembryo to the four-celled true embryo + suspensor stage. The number of endosperm nuclei associated with crossbred embryos in each of the nine instances was higher than the number accompanying inbred embryos. For example, at the six-celled proembryo stage 27.6 endosperm nuclei, on the average, were present in the selfed seeds and 41.0 in the crossed seeds. The corresponding values at the four-celled true embryo stage were 64.0 and 83.7. Thus it is seen that the endosperm in *Medicago sativa* grows faster after outcrossing than after selfing and that a crossbred embryo at a given early stage of development is accompanied by a more advanced endosperm than is an inbred one. The probable relation of these facts to abortion of the seed is seen when consideration is given to the changes taking place concurrently in the maternal tissue adjacent to the endosperm.

The alfalfa ovule possesses two integuments, the inner one being characteristically two cells thick. Only a small amount of nucellar

tissue at the chalazal end of the ovule remains at the mature embryo sac stage so that the endosperm, as it develops, lies in direct contact with the inner integument. Prior to fertilization the ovule is quiescent. Immediately following this event, however, not only do endosperm and embryo begin to grow but active cell division is initiated in the funiculus and integuments also. The cells of the inner integuments in the normally developing seed become highly vacuolate shortly after fertilization and assume the general appearance of tapetal cells. They continue to divide, but the plane of division is such that the integument increases in length but remains two cells in thickness.

A significant departure from the normal course of events occurs in the inner integument in the chalazal region adjacent to the funiculus at the onset of seed collapse. The cells become more densely cytoplasmic and assume a meristematic appearance. The frequency of cell division then increases abnormally. The mitotic figures are oriented at various angles and cell plates form in diverse planes so that several irregular cell layers result in place of two. It is noteworthy that these changes begin in the region near which the vascular bundle terminates and hence may be assumed to be related to the nutrient supply of the seed.

Collapse of the endosperm, which occurs within six days in about one-third of the inbred seeds, begins in the region opposite the proliferating inner integument and proceeds towards both ends of the seed. The chalazal portion of the endosperm collapses and may be completely disorganized, while the micropylar end may still be more or less turgid. The cells of the embryo in such a seed become highly vacuolate and starved in appearance. Shortly thereafter the remaining endosperm and the embryo break down.

Development of the seed in reciprocal crosses between common *Medicago sativa* ($n = 16$) and a diploid form of *M. falcata* ($n = 8$) has been studied by Ledingham (145). Two triploid hybrids were obtained following an unrecorded number of matings between a particular strain of *M. sativa*, used as the pistillate parent, and the diploid *M. falcata*. Approximately 700 flowers on other *M. sativa* plants were similarly pollinated without securing any additional hybrids. Ledingham observed that upwards of 25% of the ovules become fertile in the *M. sativa* \times diploid *M. falcata* mating and that small triploid proembryos are formed. The seeds usually collapse,

however, before five days and the fruits drop off. An occasional hybrid seed advances to the 16-celled embryo stage before abscission. The maximum number of nuclei observed in the endosperm after this mating was 16.

Fifty per cent of the ovules were found to be fertile at 32 hours after pollination following the reciprocal cross, diploid *M. falcata* \times *M. sativa*. Usually two-celled proembryos are present at this stage accompanied by eight to 16 free nuclei in the endosperm. Subsequent development is very slow and ceases altogether in many seeds at about six days. An occasional seed lives to 18 days. Triploid embryos showing early differentiation of the cotyledons were observed in some of these. Initial development of the endosperm was considered to be somewhat more rapid than in normal *M. falcata*. This pace is not maintained, however, in older seeds, and the tissue may break down completely before any sign of degeneration appears in the embryo.

Ledingham obtained 50 seeds from more than 400 diploid *M. falcata* flowers pollinated by *M. sativa*. Twenty-two plants were grown whose chromosome number was determined. Seventeen of the individuals proved to have $2n = 32$ chromosomes, and the remaining five were diploid. The tetraploids had the phenotype expected of hybrids. Presumably they arose by fertilization of unreduced *M. falcata* eggs by normal *M. sativa* sperm. Histological evidence was obtained indicating that seeds containing tetraploid embryos from the diploid *M. falcata* \times *M. sativa* cross are capable of development. Among the seeds collected at 18 days from this cross one was found which was significantly larger than any other hybrid embryo observed. It was shown to have 32 chromosomes. The endosperm surrounding this tetraploid embryo was cellular and normal in appearance. A chromosome count on the endosperm could not be obtained.

MALVACEAE

Pollen tubes enter the embryo sacs in more than half the cases in reciprocal crosses between American 26-chromosome and Asiatic 13-chromosome species of cotton (9). Ordinarily the capsules drop within 10 days after pollination when American types are used as pistillate parents in this mating but may persist for 30 days in the reciprocal cross. The seeds in the latter case, however, are

small and rarely capable of germination. The endosperms in seeds collected at seven days in the high ♀ × low ♂ cross were poorly developed, although the embryos appeared to be about normal in size. Embryos at 19 days were markedly undersized, and only traces of endosperm were present. The seeds appeared to enlarge at a normal rate after the reciprocal cross, low ♀ × high ♂. The endosperm, however, ceased development after seven to nine days, and rate of growth of the embryos, which appeared normal up to this time, then fell off sharply. Seeds at 30 days contained poorly differentiated embryos ranging from very small up to about one-quarter normal size.

Stephens (239) tested a selfed strain (N14) of *Gossypium arboreum* var. *neglectum* ($2n = 26$) and an autotetraploid ($4n = 52$) derived from it by colchicine treatment in crosses with several wild diploid species. The $2n$ strain gave empty seeds with well developed lint following pollination with *G. aridum*, *G. armourianum* and *G. raimondii*. Plump seeds were obtained in all these cases when the $4n$ strain of *G. arboreum* was used. The tetraploid on pollination with *G. klotzschianum* and *G. davidsonii* yielded empty seeds, and about 25% plump seed when mated with *G. stocksii*. The reciprocal crosses, $2n$ wild species × $4n$ *G. arboreum*, gave no plump seed. Since only one boll was set from these matings, however, apparently few ovules were fertilized.

OENOTHERACEAE

The first histological studies on collapse of the immature seed appear to have been made in an effort to account for the irregular breeding behavior of the Evening Primrose (207). This investigator had postulated that the genes in *Oenothera* are inherited as maternal and paternal sets or "complexes" rather than as freely assorting groups. He further assumed that lethal and sub-lethal factors interfered with the transmission of certain meiotic products in the gametophyte generation and that balanced lethals, operating at the seed stage, eliminated particular classes of progeny. The maintenance of certain forms as "complex-heterozygotes" was believed to rest upon this mechanism. A primary objective of Renner's histological work was identification of the inviable types of offspring in the seed. Those who may wish to relate the histological observations to the important genetic work with which

Renner coupled them are referred to the original papers and to reviews (33, 242). The present discussion will be limited to the character of the seed failure as such.

Renner (207) found that whereas the crosses *Oenothera biennis* \times *muricata* and *O. biennis* \times *Lamarckiana* gave only sound seed, all the seeds from the cross *O. muricata* (Venice) \times *biennis* collapsed at an early stage. *O. Lamarckiana* \times *biennis* gave about one-half sound and one-half empty seeds. Approximately two-thirds of the seeds from *O. Lamarckiana*, selfed, were abortive.

Double fertilization occurred normally following each of these matings. It is characteristic of *Oenothera*, however, that the endosperm nuclei are diploid rather than triploid as a result of the participation of one instead of two polar nuclei in the secondary fertilization.

Renner observed that the first evidence of abnormality in the potentially non-functional seeds from the *O. muricata* \times *biennis* cross was a marked retardation in endosperm development. This difference became evident before the embryos had advanced beyond the three- to four-celled stage and still appeared healthy. The few endosperm nuclei present also became greatly hypertrophied. Subsequent growth of the embryo became slow and ceased altogether at about the 20-celled stage. Later the embryo sometimes disappeared. The seed coat, however, developed relatively well. The nucellar cells in the chalazal region of collapsing seeds several weeks old proliferated, a phenomenon which Renner never observed in normal seeds. Defective seeds resulting from the *O. Lamarckiana* \times *biennis* mating showed similar relations. More than eight nuclei in the endosperm were rarely seen and these were usually of enormous size. The embryo cells were large, deficient in cytoplasm, and increased in number very slowly.

Hiorth (103) made a series of matings designed to yield, in each case, a lethal-carrying "complex" in homozygous condition, in addition to normal seeds. The course of development of the two classes of seeds was then studied histologically. He confirmed Renner's observation that the endosperms of aborting seeds were weakly developed. Giant nuclei frequently occurred. Certain genetic classes of zygotes were found not to divide at all. Others formed multicellular embryos before growth was arrested. The cells in embryo and endosperm of collapsing seeds were poor in

cytoplasm. The seed coats, on the other hand, appeared to grow normally.

A histological study of seed development in *Epilobium* was made (168) in an effort to determine why hybrids between species in the sections Chamaenerion and Lysimachion could not be obtained. Fertilization was found to be normal in crosses between these two groups, but the embryos ceased growth prematurely. Hybrid embryos from crosses involving Chamaenerion species as pistillate parent tended to fall into two equally frequent groups. One class of embryos failed very early, whereas the other continued to develop to 14 days or longer and then collapsed. Various irregularities in embryo development were observed. Sometimes loosely articulated masses of 15 to 20 cells were formed. Multinucleate cells were occasionally produced. The cells were poor in cytoplasm and the nuclei weakly stainable. The endosperm nuclei, "which occur in only very limited numbers", were greatly enlarged and reacted feebly to stains. Proliferation of nucellar tissue occurred in association with the weak endosperm development. Individual nucellar cells, which sometimes gave rise by division to two- to eight-celled structures, extended into the gametophyte cavity and often exceeded the embryo in size. Michaelis points out that these cell growths recall adventive embryos and agree with the female gametophyte picture which Haberlandt (92) obtained upon wounding the ovary in *Oenothera*. He concludes, however, that these outgrowths are not embryos.

LABIATAE

The synthetic form of *Galeopsis Tetrahit* ($2n = 32$), which Müntzing (174) obtained from *G. pubescens* ($2n = 16$) and *G. speciosa* ($2n = 16$) in his now classical experiment on the artificial production of a Linnean species, was shown to give a normal complement of seed on mating with the standard *G. Tetrahit* and the closely related *G. bifida*. No functional seeds were formed, however, on pollinating the artificial *Tetrahit* with the parent species, *pubescens* and *speciosa*. Fertilization occurs, and endosperm and embryo begin to develop normally. Growth slows down, however, at 10 to 12 days. Two days later seeds were observed in which the endosperm was breaking down, although the embryo still appeared sound.

SOLANACEAE

Fertilization and development of the resulting hybrid seeds occurs for varying but limited periods of time after the following matings between species of *Nicotiana*: *N. Rusbyi* \times *N. Langsdorffii*, *N. rustica* \times *N. longiflora*, *N. tabacum* \times *N. Langsdorffii*, *N. paniculata* \times *N. suaveolens*, *N. rustica* \times *N. glauca*, *N. rustica* \times *N. Rusbyi* and *N. rustica* \times *N. glutinosa* (132). Embryos comprising four to six cells are also formed after application of *Petunia violacea* pollen to *Nicotiana paniculata*, *N. glauca* and *N. suaveolens*. Germinable seeds, however, do not develop in any of these cases. Occasional seeds from the cross *N. rustica* \times *N. tabacum*, on the other hand, are capable of sprouting and give rise to viable plants. A histological examination of the *N. rustica* \times *N. tabacum* seeds showed that the embryos begin to develop normally but, compared with *N. rustica* selfed, soon show a marked retardation of growth. Kostoff noted also that overgrowth of the nucellus, particularly in the chalazal region, commonly occurred in the hybrid seeds. Starch accumulates in the integument in excessive amounts.

The abnormal seed development in the cross *Datura stramonium* ($n = 12$) \times *D. metel* ($n = 12$) has been studied histologically (213). Fertilization occurs in the hybrid mating about 48 hours after pollination in contrast with 24 hours for normal *D. stramonium* used as the control. Detailed examination of the fertilization process failed to disclose any difference in behavior in the two cases. Early growth of endosperm and embryo was very similar in the hybrid and normal seeds. The rate of growth of the hybrid seeds is retarded so that they have fallen behind the normal seeds within six to seven days. The hybrid endosperm has from 18 to 30 cells, whereas that of *D. stramonium* is comprised of 80 to 90 cells at this time. The proembryo of the cross consists of four to six cells as compared with 16 to 30 cells in the control embryo. The differences in size of endosperm and embryo increase rapidly as the seed becomes older due to continued growth of the control and a gradual arrest of development of the hybrid seed.

Satina and Blakeslee report that disintegration occurs in some hybrid seeds at six days but is delayed in others until 13 days. The epithelial cells surrounding the endosperm are greatly increased in size in the collapsing seeds. Breakdown was believed to occur independently in the endosperm and the embryo.

A detailed histological study of seed development following the mating *Nicotiana rustica* \times *N. glutinosa* has also been made (38). Although fertilization takes place, germinable hybrid seeds are not formed. Fertilization occurs about 60 hours after pollination in the cross as compared with about 24 hours in *N. rustica* selfed. The endosperm consists of 16 to 24 large and highly vacuolated cells at the time the zygote divides in the normal *N. rustica* seed about four hours after fertilization. The two-celled proembryo is accompanied by 24 to 64 endosperm cells. A small group of integumentary cells opposite the antipodals (which persist for a short period in the young seed) become depleted of their contents prior to fertilization, forming the chalazal pocket. The vascular bundle which terminates in the funiculus of the ovule establishes a direct nutritive connection with the chalazal pocket in the day-old seed by differentiation of the intervening cells into a conducting tissue. Growth of the seed now proceeds rapidly. Many hundreds of large vacuolate cells are present in the endosperm at 144 hours, and the embryo comprises a spherical mass of densely cytoplasmic cells.

The endothelium (referred to as nucellus, possibly incorrectly, in the original paper) comprises a single layer of cells in the ovule which completely surrounds the female gametophyte except for a gap opposite the chalazal pocket. The endothelium remains one layer of cells in thickness as the normal *N. rustica* seed develops. Growth occurs by cell division as the endosperm expands. The individual cells become flattened between the endosperm and the surrounding mass of integumentary tissue. The first evidence of cotyledon formation in the embryo is seen at about 15 days. A few starch grains appear in the integument, although not in any other part of the seed, at this time.

Nuclear and cell divisions in the endosperm of the young *N. rustica* \times *N. glutinosa* seed proceed more slowly than in the normal *N. rustica* seed. The cells become smaller as they increase in number, and the cytoplasm after each succeeding division is less vacuolate. The number of cells in the endosperm varies from eight to 16 at the time the hybrid zygote divides. Two-celled proembryos are accompanied by endosperms possessing 16 to 48 cells. The integumentary cells between the vascular bundle and the chalazal pocket fail to become differentiated into conducting elements.

A striking transformation occurs in the endothelium of the young hybrid seed. Beginning in most cases when the endosperm is eight-celled, but occasionally earlier, the endothelial cells enlarge and soon become actively meristematic. The meristematic activity is initiated in the cells nearest the chalazal region and on the funicular side of the seed opposite the vascular bundle. The plane of division of the endothelial cells is frequently parallel to the long axis of the seed so that a tissue several cell-layers in thickness is formed. Continued proliferation of the endothelium in the hybrid seed often results in the formation of a large and irregular mass of cells in contrast with the orderly development of this tissue as a single cell-layer in the normal seed.

The first evidence of collapse of the hybrid seed appears in the chalazal end of the endosperm. This region is adjacent to that in which hyperplasia of the endothelium is initiated. The breakdown of endosperm cells progresses toward the embryo end until eventually the entire tissue collapses. Starch is stored in significant amounts in the endothelium and seed coat of the aborting seeds. Meanwhile the embryo shows signs of starvation, but actual breakdown of this structure does not occur until the endosperm has disintegrated.

The average total volume of endosperm plus endothelium at the 20-celled embryo stage was found to be about five times as high in *N. rustica* selfed seeds as in the hybrid seeds. Furthermore 77% of the volume was occupied by endosperm tissue in the normal seed and only 25% in the other class.

Most hybrid seeds cease growth by six days. Occasionally one is found that is still developing at nine days. Extensive overgrowth of the endothelium occurs in these longer lived hybrid seeds, and the endosperms are much smaller and have denser cells than normal *N. rustica* seeds. A regular feature is a finger-like projection of the endosperm through the endothelial aperture into the chalazal pocket. This circumstance, together with the fact that numerous integumentary cells adjacent to the chalazal pocket lose their contents, suggests that these more persistent seeds owe their advantage to a somewhat better nutrition of the endosperm.

The writers (19) observed a similar series of events leading to collapse at the two-to four-celled embryo stage of the *Nicotiana rustica* \times *Petunia violacea* seed. A limited growth of the endosperm

was associated with a pronounced hyperplasia of the endothelium. No evidence was found for the stimulation of ovular growth in the absence of fertilization, such as Kostoff (132) reported.

Fertilization was observed to occur at about 96 hours after pollination of *Nicotiana rustica* by *Lycopersicon esculentum*. The fertilized egg does not divide, but development of the endosperm may proceed to the eight-celled stage. The endothelium in collapsing seeds is in a meristematic condition.

The cross *Nicotiana rustica* \times *N. tabacum* yields small shrunken seeds a few of which, however, are germinable. Two F_1 plants were vigorous, intermediate between the parents in habit of growth, and slightly fertile (31). The deleterious effect on seed development of this interspecific mating, therefore, is less extreme than that of the *N. rustica* \times *N. glutinosa* cross in which all the seeds die early. Brink and Cooper (20) sought to determine the histological basis of the difference.

The course of development of the *N. rustica* \times *N. tabacum* seeds parallels that in the *N. rustica* \times *N. glutinosa* mating in that (a) endosperm growth is retarded, (b) pronounced hyperplasia of the endothelium occurs, and (c) the integumentary cells lying between the apex of the vascular bundle and the chalazal pocket fail to differentiate into conducting elements. The difference between the two classes of seeds with reference to (a) and (b) is quantitative, the *N. tabacum* hybrid showing a less abnormal development than the *N. glutinosa* hybrid. For example, the volume ratio of endosperm to endothelium at the two-celled proembryo stage was 1.47 for *N. rustica* selfed, 0.72 for *N. rustica* \times *N. tabacum* and only 0.51 for *N. rustica* \times *N. glutinosa*.

Growth of the endosperm and embryo in the *N. rustica* \times *N. tabacum* seed is at the direct expense of the integument. The endosperm of the longer persisting seeds continues to maintain a connection with the chalazal pocket through the aperture in the overgrown endothelium. The endothelial cells adjacent to the chalazal pocket lose their contents rather early, and, as the seeds become older, a large part of the integument becomes depleted. In the normal *N. rustica* seed, differentiation into conducting tissue of the cells between the apex of the vascular bundle and the chalazal pocket enables a portion of the nutrients moving into the seed to pass directly to the endosperm. The latter nourishes the embryo which it

encloses. Presumably nutrients also diffuse from the conducting cells into the integument, supporting growth in this tissue, including the endothelium. The differentiation does not occur in the hybrid seeds, however, so that the conducting element terminates in the funiculus. The integument thus becomes the immediate recipient of all the incoming food materials. Functionally, the endosperm then assumes the rôle of an haustorium, absorbing nutrients from the integument. The integumentary cells are unable to replace fully the materials thus taken up by the endosperm and become progressively depleted as development continues. Most of the *N. rustica* \times *N. tabacum* seeds fail under these conditions, but an occasional one is able to develop to a germinable stage.

In the $3n \times 2n$ mating in *Datura stramonium* elimination of female gametes and of zygotes at various stages of development reduced the number of seeds in the capsule to 61 as compared with approximately 10 times this number for the $2n$ plant, normally pollinated (214). A total of 273 offspring of the $3n \times 2n$ cross were examined cytologically. Forty-eight per cent of these were $2n+1$, 20% were $2n$, 28% were $2n+1+1$ and 3.5% were $2n+1+1+1$. Thus the only eggs from the triploid individual which are represented as offspring living to a recordable age are those having n , $n+1$, $n+1+1$ and $n+1+1+1$ chromosomes. Examination of the entire complement of one ovary six days after pollination showed that only 124 of the several hundred ovules present were fertilized. Likewise only 21% of the ovules in five ovaries fixed seven days after pollination were enlarged. The corresponding value for normal diploids was 87. Thus the frequency of fertilization in the triploid is low. Observations on dividing nuclei in the endosperm of seeds from the $3n \times 2n$ mating revealed, however, that gametes with all chromosome numbers from 12 to 24 with the exception of 19, 23 and 24 had participated in fertilization. It is believed that the missing numbers would have been found had more seeds been examined. Evidently there is a pronounced differential elimination of the unbalanced chromosomal types subsequent to fertilization.

Considerable variation in rate of early seed development was observed in the $3n \times 2n$ cross. Some seeds were found at six to eight days after pollination with undivided zygotes and small endosperms. Others showed embryos with 16 to 20 cells and endosperms with over 300 cells. A great diversity of conditions with reference to

embryo, endosperm and testa development was found in the seeds which were obviously defective at maturity. No traces of an embryo could be found in some seeds, even though normal endosperm tissue was present. Conversely the endosperm might be absent or much reduced in size in seeds having a well differentiated embryo. The embryos were usually undersized and frequently misshapen.

RUBIACEAE

The species *Galium Mollugo* comprises diploid, tetraploid and hexaploid forms, the basic chromosome number being $n = 11$. Fagerlind (72) observed that whereas $2n \times 2n$ and $4n \times 4n$ matings were fertile, $4n \times 2n$ crosses yielded no germinable seeds, and that $2n \times 4n$, $2n \times 6n$ and $4n \times 6n$ matings rarely gave them. Fertilization occurs freely in the $4n \times 2n$ combination, but a lag in endosperm development is noticeable at two days and becomes pronounced at seven days. The onset of cell wall and haustorium formation in the young endosperm, however, appears not to be delayed. The embryo at seven days is somewhat smaller than normal. Most seeds collapse by 14 days. Development of endosperm and embryo is much more variable in the $2n \times 4n$, $2n \times 6n$ and $4n \times 6n$ matings. The endosperm is usually voluminous at seven days and is comprised of cells varying widely in chromosome number. Wall formation is often irregular after endosperm cell division. Nuclear fusions, sometimes involving numerous nuclei, are frequent. It is of interest to note that *Galium* has specialized antipodals which persist long after the endosperm has become cellular. The latter tissue in the normal seed eventually surrounds the antipodals completely.

DIPLOID-AUTOTETRAPLOID CROSSES

The seeds arising from reciprocal crosses between diploid plants and their respective autotetraploids are usually abortive and only rarely capable of germination. Attention was called to this fact in the $4n \times 2n$ mating of *Datura stramonium* (12), and the same relation was shown to obtain in *Lycopersicon esculentum* (115). Numerous additional examples have since been observed. In the $2n \times 4n$ mating in *Datura stramonium*, fertilization seldom occurs due to faulty development of diploid pollen tubes in diploid stylar tissue (26). If fertilization is accomplished in the $2n \times 4n$ combination, the seed usually breaks down, just as in the reciprocal cross.

The evolutionary significance of the reproductive isolation of tetraploids from their diploid progenitors has been widely recognized. The limited investigations which have thus far been made on the cellular physiology of polyploidy do not afford an explanation of the remarkable fact that doubling of chromosome number alone, in the absence of qualitative gene differences, establishes a barrier to effective hybridization between these forms. Some evidence concerning the tissue relations involved in these cases of seed failure, however, is forthcoming from histological studies (212) and more recently from work on diploids and tetraploids carrying similar genes (41).

Using the proportion of ovules enlarged at seven to 14 days after pollination as the basis of their estimate, some investigators (212) found that, on the average, about two-thirds of the ovules in a capsule are fertilized after the $4n \times 2n$ mating in *Datura stramonium*. The $4n \times 4n$ combination gave a slightly lower value. Only about 1% of the ovules in the $4n \times 2n$ cross, however, are represented in the mature capsule as large seeds, and many of these are incapable of germination. About 25% of the ovules in the $4n \times 4n$ capsule, on the other hand, yield well developed seeds.

Division of the generative nucleus in the pollen tube was shown to take place normally in the $4n \times 2n$ cross. Nor were any irregularities observed in the process of double fertilization which would account for the subsequent failure of the seeds to develop. Nineteen out of 20 endosperm nuclear divisions examined showed 60 chromosomes, the expected number, and one had 48 chromosomes. Embryo divisions were found with difficulty, but of six which permitted a chromosome count, four were triploid, one was estimated to be approximately triploid and a sixth appeared to be diploid. No evidence of irregularity in the distribution of the chromosomes in nuclear division, in either endosperm or embryo, is reported.

The endosperm in $4n \times 2n$ *Datura* seeds may start to disintegrate when they reach the 30- to 40-cell stage. Relatively few proembryos develop beyond the four-cell stage. In conjunction with the breakdown of endosperm and embryo, the endothelial cells enlarge and may divide periclinally, forming a multi-layered tissue. The proliferating endothelium frequently fills up the embryo sac. The early stages of development of the $2n \times 4n$ seed appeared to be essentially like those of the $4n \times 2n$ seed.

No difference was found in the rate of endosperm development previous to division of the zygote in $4n \times 2n$ and $2n \times 4n$ seeds as compared with the $4n \times 4n$ control. At the two- to six-cell proembryo stage, however, the $4n \times 2n$ and $2n \times 4n$ seeds were found to have about 50 cells in the endosperm, on the average, as compared with about 120 for the control seed.

Breakdown, once begun, was found to proceed very rapidly, so that seeds showing disintegration of only a part of the endosperm were rarely observed. Although it appears likely that collapse usually begins in the endosperm, Sansome, Satina and Blakeslee do not consider that the evidence from the *Datura* material for such a conclusion is unequivocal.

Cooper and Brink (41) have studied the nature of the block to continued development of the seed following the crossing of practically isogenic diploid ($2n = 24$) and tetraploid ($2n = 48$) races of *Lycopersicon pimpinellifolium*. Matings between the $2n$ and $4n$ strains of *L. pimpinellifolium* (referred to subsequently merely as $2n$ and $4n$, resp.) with *L. peruvianum* ($2n = 24$) as pollen parent were included so as to give a direct comparison between seed failure associated with a difference in chromosome number and that resulting from a species cross. The controls used were *L. pimpinellifolium* seeds from $2n \times 2n$ and $4n \times 4n$ matings.

Over 90% of the pollinated flowers in the $2n \times 2n$ and $2n \times L. peruvianum$ crosses gave rise to fruits which developed to maturity. The $2n \times 2n$ fruits contained a normal complement of plump seeds (about 27, on the average). The $2n L. pimpinellifolium \times L. peruvianum$ mating gives abortive seeds.

The $2n \times 4n$ mating is barren, the flowers and young fruits all falling within eight days after pollination. A few ovules become fertile, however, and observations were made on early development of the seeds.

About 55% of the flowers pollinated in the $4n \times 4n$ mating formed mature fruits. These contained, on the average, eight plump and approximately four shrunken seeds each. The frequency of fertilization, estimated from the proportion of ovules enlarged at 12 days, was found to be only about 20%. About three times as many ovules become fertile in the $4n \times 2n$ mating as in the $4n \times 4n$ combination, and 75% of the fruits grow to maturity. The seeds within these fruits, however, are abortive and non-functional.

The $4n \times L. peruvianum$ mating yielded many fruits containing an average of 16 small plump and 11 shrunk seeds each. A limited test indicated that the plump seeds are viable, and the two triploid hybrid plants reared were exceptionally vigorous. This interspecific mating, therefore, is much more fertile than the $4n \times 2n$ intraspecific combination.

The rate of early endosperm and embryo growth in the seeds resulting from these various matings was measured by counting the number of nuclei in serial sections at 24, 48, 96, 144 and 192 hours after pollination.

The endosperm in $2n \times 4n$ seeds makes a very weak growth, lagging far behind the control, $2n \times 2n$, from the start. Endosperm growth in $2n \times L. peruvianum$ seed is faster than that in the $2n \times 4n$ cross but still much inferior to the control.

Development of the endosperm in the $4n \times 2n$ cross, similarly, is markedly inferior to that in the $4n \times 4n$ control. Assuming that fertilization occurs at the same interval after pollination, the initial rate of growth of the $4n \times L. peruvianum$ endosperm is higher than that in the $4n \times 4n$ seed. A rapid acceleration of growth in the $4n \times 4n$ endosperm then follows. These data indicate that the rates of endosperm growth in the $2n \times 4n$ and $4n \times 2n$ seeds, whose development is arrested in the young fruit, are markedly lower than those of the respective controls. The $2n \times L. peruvianum$ endosperm is also weak. The $4n \times L. peruvianum$ endosperm is definitely inferior to that of the $4n \times 4n$ seed, but it is nearly three times the size of the $4n \times 2n$ tissue at 192 hours. It will be recalled that the $4n \times L. peruvianum$ fruits contain numerous small but apparently sound seeds, in addition to shrivelled ones.

The young *Lycopersicon* embryo grows much more slowly than the endosperm. The $2n \times 4n$ embryo is about one-half the size of the $2n \times 2n$ control at 144 hours, and usually ceases growth shortly thereafter. The $2n \times L. peruvianum$ embryos are somewhat smaller than $2n \times 2n$ embryos at 192 hours. Similarly the $4n \times 2n$ and $4n \times L. peruvianum$ embryos lag behind the $4n \times 4n$ class. There seems to be little direct relation, however, between seed survival and size of embryo at this early stage. The $4n \times L. peruvianum$ embryo at 192 hours, for example, is considerably smaller than the $4n \times 2n$ embryo. Yet seeds of the latter class all fail early, whereas many of the former develop to maturity.

The integumentary cells between the apex of the vascular bundle and the chalazal pocket in $2n \times 2n$ and $4n \times 4n$ seeds are differentiated into conducting cells during the period between fertilization and 144 hours. This differentiation is less complete in $2n \times 4n$ and $4n \times 2n$ seeds. The latter also show an accumulation of granular material of unknown character in the chalazal pocket. A few to several layers of integumentary cells immediately outside the endosperm lose their cytoplasmic contents during early development of the seed. This depletion proceeds less rapidly in the $2n \times 4n$ and $4n \times 2n$ seeds than in the respective controls. A marked enlargement of the endothelial cells lying along the portion of the dorsal surface of the endosperm nearest the chalaza appears in $2n \times 4n$ seeds at 144 hours. The corresponding cells in $4n \times 2n$ seeds are beginning to proliferate at this time. The endosperm cells adjacent to the overgrown portion of the endothelium become very poor in cytoplasm. Disorganization of the endosperm now appears in many $2n \times 4n$ and $4n \times 2n$ seeds. All the seeds of the former class break down well before 288 hours, and the fruits containing them fall. The endosperm in the $4n \times 2n$ seeds has disintegrated entirely by this time and the embryo is surrounded by a mass of overgrown endothelial tissue.

The $2n \times L. peruvianum$ seeds, all of which collapse eventually, follow a similar course of development except in one respect. Beginning near the apex of the embryo the endosperm cell walls begin to break down. The nuclei of the fusing cells unite, so that giant nuclei with numerous nucleoli are formed. The dissolution of cell walls and fusion of nuclei continues until the endosperm collapses and growth of the embryo and seed ceases.

Many $4n \times L. peruvianum$ seeds, on the other hand, develop in a comparatively normal manner. Endosperm growth is slower than in $4n \times 4n$ seeds, but the endothelium rarely becomes more than a single cell-layer in thickness. At 16 days, however, the $4n \times L. peruvianum$ endosperm contains no granular food reserves in contrast with an abundance in the $4n \times 4n$ seeds. A limited disorganization of the endosperm cells adjacent to the endothelium and digestion by the embryo of a relatively large central portion of the endosperm appears to be associated with a scarcity of nutrient materials.

(To be concluded in next issue)

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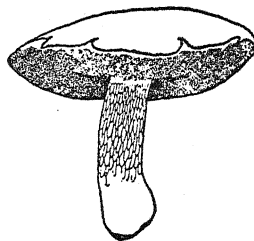
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THE ENDOSPERM IN SEED DEVELOPMENT

(Concluded)

R. A. BRINK AND D. C. COOPER

University of Wisconsin

CAUSES OF ARRESTED SEED DEVELOPMENT

The ideas advanced to explain the occurrence of shrunken and aborted seeds will be considered under two headings, genetic relations and developmental conditions. The first deals with the hereditary prerequisites for seed collapse; the second is concerned with the tissue changes leading to breakdown. The two aspects of the problem are closely related and cannot be treated entirely apart from each other. Since genes are perceived only by their modes of action, an hereditary analysis requires that the phenomenon being dealt with be characterized developmentally in some degree. Such characterization in the case of seed collapse requires, in the first instance, that the site of the primary departure from normal growth be identified. Since both endosperm and embryo are products of fertilization, the answer to this problem is not self-evident but must be sought in the behavior during development of these two tissues. Endosperm and embryo grow in intimate association with each other and with the maternal parts of the seed. All three tissues are affected in aborting seeds. An interpretation of the mechanics of seed failure, therefore, must account for the nature and sequence of changes occurring in these correlated structures.

Genetic Relations

Several kinds of hereditary alteration in balance between endosperm, embryo and maternal tissue are known to lead to seed abortion.

(a) Enforced self-fertilization in a normally cross-fertilized species: *e.g.*, *Pyrus* (265), *Zea* (148, 159), *Secale* (140) and *Medicago* (19).

Lindstrom (148) found that two cases of defective seeds in maize were inherited as simple recessives. Other extensive tests (159) indicated that in open-pollinated varieties of maize about one plant in 30 is heterozygous, for one or another recessive gene conditioning defective seed development is widespread in maize. Brink and Cooper (19) suggested that the reduced rate of early endosperm growth following enforced self-fertilization in *Medicago sativa* is an inbreeding depression phenomenon associated with the increased homozygosity for numerous recessive genes, each having a small adverse effect on development. They also suggested the same explanation for the facts which Landes (140) has reported for *Secale cereale*. Breeders are familiar with the fact that continued self-fertilization of *Zea mays*, aside from bringing to light numerous markedly defective seed types, reduces the size of the functional seeds. Conversely, increased size of seed on inbred plants is a frequently occurring immediate response to outcrossing. This phenomenon appears to be a manifestation of hybrid vigor in embryo and endosperm similar to that appearing in the growing plant. Small variations in seed development, depending directly upon genetic constitution of endosperm and embryo, may be widespread in the flowering plants. The principal variable, however, may be rate of development, rather than size at maturity, as shown by unpublished data on *Medicago sativa* from this laboratory and as Buchholz (25) has found in certain gymnosperms.

(b) Crossing of species not differing in chromosome number: e.g., *Linum perenne* \times *L. austriacum* (136), *Datura stramonium* \times *D. metel* (213) and *Hordeum vulgare* \times *Secale cereale* (21, 251).

Seed failure in this class results from combining qualitatively diverse genomes in the seed. The writers are not aware of any attempt to analyze genetically a species hybrid complex whose elements interact to cause seed collapse. Inherent differences between varieties within one species to yield hybrid seeds on outcrossing to another species with the same or a different chromosome number, however, have been observed in a number of cases. Armstrong (5) found that pollen of one strain of *Agropyron elongatum*, on the average, was about three times as effective as pollen of another in causing seeds to form when applied to two varieties of tetraploid and three varieties of hexaploid wheat. The same *elongatum* strain, however, was not superior in all the combinations. Smith (228)

observed that when different varieties of common wheat were mated with *Agropyron elongatum* the percentage of florets forming seeds varied from 34 for White Odessa-Hohenheimer to zero for Marquis and Regenerated Defiance. Variations in frequency of fertilization may be involved here as well as differences in capacity for development of the respective hybrid seeds. Boyes and Thompson (15) observed that application of *Secale* pollen to Marquis wheat does not lead to fertilization, whereas the Chinese variety of wheat hybridizes readily when pollinated with rye to give shrunken, although germinable, seeds.

(c) Crossing of races differing essentially only in degree of polyploidy: e.g., $4n \times 2n$ and $2n \times 4n$ matings in *Datura stramonium* (212), *Zea mays* (201) and *Lycopersicon pimpinellifolium* (41).

Matings of $4n \times 2n$ in *Datura stramonium* have given rise to abortive seeds (12), and similar results with other autotetraploids have since been obtained, showing that purely quantitative changes in the 2:3:2 chromosomal ratio of maternal tissue, endosperm and embryo is an important cause of seed collapse. Seed abortion associated with differences in degree of polyploidy between intra-specific races is of particular interest because the sterility gives a basis for evolutionary divergence of initially isogenic types. The phenomenon, accordingly, has received considerable notice. Discussion of the subject, however, has usually been related to crosses between species varying not only in degree of polyploidy but also in genic composition. Such matings properly belong to a category different from (c) above, namely,

(d) Crossing of species differing in both degree of polyploidy and genic complement: e.g., *Avena strigosa* \times *A. fatua* and reciprocal (130), and *Nicotiana rustica* \times *N. glutinosa* (38).

Several investigators have recognized the difficulty in cases of this kind of evaluating the effect on seed development of polyploidy alone. Cooper and Brink (41) found that quantitative and qualitative factors in tomato are not simply additive in their effects on the seed but may interact to give results which are not predictable from the action of each variable operating singly. The $2n \times 2n$ mating in *Lycopersicon pimpinellifolium* is fully fertile, whereas the $4n \times 2n$ and $2n \times 2n$ *L. peruvianum* crosses give all abortive seeds. Contrary to what would be expected from these facts on the additive basis, tetraploid *L. pimpinellifolium* when pollinated by diploid *L.*

peruvianum yields seeds, many of which, although small, are germinable. The regular occurrence of seed abortion in $4n \times 2n$ intraspecific matings has been proven, so that it is reasonable to assume that the same factor is operative in corresponding species crosses. At the same time it is clear that the expression in interspecific hybrid seeds of a polyploidy effect may be more or less profoundly modified by concomitant gene differences in the parents.

Watkins (267), working with *Triticum vulgare* ($n=21$), *T. turgidum* ($n=14$) and the F_1 hybrid between them, observed that germination of the seeds is good following matings giving endosperms in which the seven extra *vulgare* chromosomes are either diploid or triploid, and poor in the cases in which some or all of these chromosomes are only haploid. Further studies led Watkins (268) to the conclusion that it is the quantitative relation in chromosome number between endosperm and embryo which is significant in seed development and that the chromosome number of the maternal parent is unimportant. Müntzing (177) takes issue with Watkins on the latter point on the grounds that female gametes of a given kind may function in seed development or not depending upon the constitution of the plant giving rise to them. He cites Karpechenko's (123) observation that the diploid *Raphanus* \times *Brassica* F_1 hybrid forms seed on being pollinated with the *Raphanus* parent. The corresponding mating involving the amphidiploid *Raphano-brassica*, however, is rarely fruitful. The functional gametes in both cases have the same chromosome number but are borne by a diploid plant in one case and a tetraploid in the other. Müntzing's point of view is further supported by his observation that in *Galeopsis* $4n \times 2n$ matings result in abortive seeds. Unreduced eggs produced by $2n$ plants and fertilized by n sperm, on the other hand, may give viable offspring (174).

Others (15, 247, 250) have brought forward considerable additional evidence demonstrating the importance of quantitative chromosomal differences in the endosperm for seed formation in the cereals. Thompson (248) has marshalled the data available from the Gramineae and other families, showing that in crosses between species having different chromosome numbers in the same multiple series viable seeds are more likely to be formed if the species with the larger number serves as the pistillate parent. Although exceptions have been encountered this generalization appears to have

a wide validity. The difference in seed setting following such reciprocal crosses was believed by Thompson to be due to the fact that when the species with the larger number is the pistillate parent the excess of its chromosomes over those of the other species will be doubled in the endosperm, whereas when it is the staminate parent they will be single.

The results of backcrosses between allopolyploid *Galeopsis* plants and their parent species and of matings between autopolyploids and their parents led Müntzing (174) to conclude that the principal reason for poor hybrid seed development is disturbance of the normal 2:3:2 ratio of chromosome numbers of embryo, endosperm and maternal tissues. Müntzing emphasizes that it is not the number and quality of the chromosomes in the endosperm as such which is of prime importance for growth of the seed, but rather the balance in number in embryo, endosperm and associated tissues of the mother plant.

East (66) suggested that in crosses between species differing in chromosome number the greater success of high ♀ × low ♂ as compared with low ♀ × high ♂ matings is associated with allopolyploidy. It was presumed that if the parent with the high chromosome number is an allopolyploid it would be more versatile than its more simply constituted relatives in capacity to nourish the seed. As Boyes and Thompson (15) have pointed out, however, this explanation breaks down in the case of diploid-autotetraploid crosses.

An idea somewhat similar in its implication to that of East has been put forward by Janaki-Ammal (106). The cultivated sugar cane, *Saccharum officinarum*, is typically octoploid, $2n = 80$. *S. spontaneum* is polymorphic, $2n = 48, 56, 64, 72, 80, 96, 112$. A hybrid having 66 chromosomes was obtained from the cross *S. spontaneum* ($2n = 112$) ♀ × *Erianthus ravennae* ($2n = 20 + \text{fragment}$). Several true hybrids with 120 to 134 chromosomes resulted from the mating *S. officinarum* POJ 2725 ($2n = 106$) ♀ × *Imperata cylindrica* ($2n = 20$) ♂. It is believed that the latter hybrids were the product of unreduced gametes of POJ 2725 combined with haploid gametes of *Imperata*. Equally remarkable is Janaki-Ammal's success in obtaining a viable hybrid with $2n = 52$ chromosomes from pollinating *S. officinarum* var. Villai ($2n = 80$) with *Zea mays* var. Golden Beauty ($2n = 20 + 2B$). Janaki-Ammal concludes that: "It appears that in *Saccharum* high polyploidy has

removed all obstacles to hybridization with other groups of Gramineae except those that depend upon the simply ascertainable conditions of pollen germination".

Kihara and Nishiyama (130) conclude from a study of interspecific hybridization in *Avena* that seed development depends upon the strength of stimulus to development of the male nuclei. They distinguished four grades in stimulative strength—overstrong, normal, weak and no affinity (non-fertilization)—on the basis of growth rates of endosperm and embryo following various crosses between *Avena* species with $n=7$, 14 and 21 chromosomes. Male nuclei having a chromosome number higher than normal were assumed to possess excessive "stimulative strength", resulting in abnormal rapidity of cell division in endosperm and embryo. A lower chromosome number than normal in the male gamete was believed to cause a retarded growth of embryo and endosperm. Kihara and Nishiyama attributed the abnormal nuclear division in the endosperm of $2n \times 6n$ *Avena* matings to the excessive stimulus of the high chromosome male gamete. Müntzing (177) points out that there is no direct evidence supporting the view of Kihara and Nishiyama that a sperm with a higher chromosome number than normal can cause an excessive rate of cell division in embryo and endosperm. Cooper and Brink (40) found that the rate of endosperm nuclear division was markedly lowered rather than raised in $2n \times 4n$ *Lycopersicon* crosses in comparison with the $2n \times 2n$ control. It appears doubtful, therefore, that excessive rates of cell division in the endosperm are characteristic of matings of the type in question.

Stephens (239) suggests that the main factor affecting seed development following various $2n \times 4n$ and $4n \times 2n$ interspecific matings in cotton is the genom ratio between zygote and endosperm. This is normally 2:3 and is altered to 3:4 and 3:5 in reciprocal crosses between diploids and tetraploids. Numerical adjustments in the ratio, in accordance with assumed differences in "strength" of the genomes involved, were tried in an attempt to account for the variation in seed development in a series of *Gossypium* interspecific crosses. Only a part of the cases could be explained on this basis.

(e) Aneuploidy: e.g., *Triticum* (247), *Datura stramonium* (214) and *Secale* (178).

Thompson (247) found that unbalanced chromosome conditions

in the endosperm leading to shrivelling of the caryopsis played a large part in the non-appearance of many types of plants in F_2 and later generations of matings between tetraploid and hexaploid wheats. Shrivelling of the seed was the more severe the farther the chromosome condition in the endosperm departed from the norm of the one or the other parental race. The shrivelled seeds represent cytological and genetical classes not found among the plump ones.

The number of $2n$ (24-chromosome) offspring of triploid *Datura stramonium* plants pollinated by diploids was estimated (214) to be about 800 times that expected on random assortment of the chromosomes in meiosis and random survival of the resulting female gametophytes, seeds and seedlings. The investigators found that inability to develop good seeds was an important source of elimination of zygotes with heteroploid chromosome numbers. Although relatively few ovules are fertilized in $3n \times 2n$ matings, the female gametes in the fertilized class were found to range in chromosome number from 12 to 24. Many seeds collapse soon after fertilization. The growth of others is arrested at later stages. Wide variations in development of endosperm and embryo were observed in the defective seeds. Certain chromosomes when present as extras in $2n+1$ embryos caused the seeds to be larger, and certain other chromosomes reduced seed size. Germination was considerably delayed in some of the seeds with extra chromosomes.

A small proportion of aneuploid individuals occur among the offspring of autotetraploid *Secale* plants. Müntzing (178) found that poorly developed seeds gave a higher proportion of plants with aberrant chromosome numbers than did plump seeds. The seeds containing hypotetraploid embryos were less well developed, on the average, than those giving rise to hypertetraploid plants.

(f) Maternal genotype causes arrested growth of the seed regardless of the source of the sperm participating in fertilization: *e.g.*, early maturing, cultivated varieties of *Prunus* (254) and certain "seedless" varieties of grapes (192, 195, 196, 241).

Pearson (195, 196) distinguished two classes of seedlessness in *Vitis vinifera*. In the one type of grape, exemplified by the Black Corinth variety, functional female gametophytes are not formed, or only very rarely. Fertilization, therefore, does not occur, and fruit development is parthenocarpic. The second class, represented by

the Sultanina, Sultanina Rose and Black Monukka varieties, develop normal female gametophytes which are fertilized regularly. Growth of the seeds is arrested at an early stage, but the fruits continue development. Olmo (192) observed that seed abortion in the latter case is governed by the maternal genotype and is independent of the source of the pollen. Stout (241) obtained numerous F_1 seedlings using pollen of seedless varieties of the Black Corinth class on various seeded grapes. All the offspring tested, with the possible exception of one, bore seeded fruits. Some plants bearing seedless and near-seedless fruits were obtained in F_1 and F_2 , however, from crosses between seeded varieties and Sultanina, Sultanina Rose and Black Monukka.

Developmental Conditions

The histological data summarized earlier afford some insight into the mechanism of seed failure. Although the evidence from many of the investigations is incomplete, and the observations have been interpreted variously by different workers, there appears to be a common pattern underlying this type of sterility. Seed collapse in the Gramineae may be left aside for the moment, since it appears to present certain special features. So far as the available evidence from other families goes, the developmental changes terminating in arrested growth of the seed seem to be basically similar. Furthermore, disturbance of the normal genetic balance between the principal tissues of the seed in any one of several ways including enforced self-pollination, crossing of certain races and species differing genetically either qualitatively or quantitatively, or in both these respects, may set in motion the same train of abnormal developmental events.

The gross histological changes which now appear to characterize the collapse of seeds in several families of plants were first delineated by Renner (207). He observed that in a definite proportion of seeds following certain matings in *Oenothera* endosperm development was retarded early. Embryo growth was not affected at first but slowed down later. The seed coat, on the other hand, developed relatively well, even though the embryo did not advance beyond a few cells. The nucellar cells in the chalazal region of collapsing seeds proliferated instead of regressing as in normal seeds. Renner concluded that since the testa in aborting seeds de-

veloped more or less regularly, failure was not due to starvation. Rather breakdown appeared to be attributable to an inherent incapacity of endosperm and embryo to grow.

Retarded growth of the endosperm in seeds developing subnormally has been noted also in *Epilobium* (168), *Iris* (215, 272), *Prunus* (16), *Galeopsis* (174), *Datura* (212, 213), *Pyrus* (24), *Gossypium* (9), *Medicago* (19), *Nicotiana* (19) and *Lycopersicon* (41).

Embryo development, in general, is also adversely affected in seeds so constituted that they are incapable of attaining a germinable condition. Most investigators who have compared the two tissues, however, agree that initial growth of the embryo is little changed relative to that of the endosperm. Size and form of the embryo in some seeds may be more or less radically altered later. In others the embryo shows only minor departures from the normal up to the time growth of the seed is arrested. The potential capacity for continued growth of the embryo in certain cases of seed collapse has been demonstrated directly by successful cultivation of the excised embryo on artificial media (*e.g.*, 9, 22, 136, 256).

Characteristic changes in the maternal tissues of collapsing seeds have been reported by several investigators since Renner first noted the phenomenon in *Oenothera*. Michaelis (168) observed a proliferation of nucellar tissue in association with weak endosperm development in *Epilobium*. Overgrowth of the endothelium was recognized (132) as occurring in interspecific hybrid seeds in *Nicotiana*, and others (212, 213) have described hyperplasia in the endothelium following interspecific *Datura* crosses and after matings between *D. stramonium* races differing in degree of polyploidy. The present writers (19) observed proliferation of the inner integument in self-fertilized *Medicago sativa* seeds beginning in the chalazal region opposite the end of the vascular bundle. The same condition was described also in *Nicotiana* hybrid seeds (19) and after $2n \times 4n$ and $4n \times 2n$ matings in *Lycopersicon* (41).

Brink and Cooper (19) attempted to develop a general theory of seed development which would account for the variations in growth associated with changes in genetic composition. The initial hypothesis grew out of observations on *Medicago sativa* in which it was found that enforced self-fertilization, substituted for normal cross-fertilization, increased five-fold the proportion of seeds which

collapsed within the first six days. Subsequent studies on seeds resulting from interspecific hybridization in *Nicotiana* (20, 38) and from matings between $2n$ and $4n$ races of *Lycopersicon* (41) served to broaden the factual basis from which inferences concerning the mechanism of seed failure might be drawn. The point of view to which these studies led the writers may be summarized in the following terms:

(a) The endosperm is primarily responsible for the maintenance of continuity in the life cycle at the early seed stage. Although the embryo is the cardinal component of the seed, in that it embodies the line of descent, this structure initially is incapable of performing certain essential growth functions. During this more or less brief period these functions are mediated for the embryo by the endosperm.

(b) The double complement of inheritance received by the endosperm from the maternal parent is an adaptation which facilitates functioning of the endosperm in its intercalary position between mother plant and embryo. There may be two factors involved in this relation: (a) "weighting" the genetic composition of the endosperm in the direction of the maternal parent which serves as host, and (b) the $3x$ condition, as such, of the endosperm nuclei may be significant physiologically in an environment of maternal and embryo cells whose nuclei are $2x$. One recalls in this connection the $4x$ condition of the infected cells in contrast with the $2x$ condition of the non-infected cells in the root nodules of Leguminosae. The work of Wipf and Cooper (280) indicates that the $4x$ - $2x$ chromosome balance between infected and non-infected cells is a regular feature in the symbiotic relation between the nitrogen-fixing bacteria and their host plants.

(c) The secondary fertilization in angiosperms is interpreted as a device tending to offset the disadvantage in reproduction associated with the extreme reduction of the female gametophyte, and to enhance the aggressiveness of the endosperm through conferring upon this tissue the physiological advantages of hybridity. The significance of these points becomes apparent when the mechanism of seed formation in the angiosperms is contrasted with that in the gymnosperms. The egg is fertilized in the latter, but the endosperm remains a haploid tissue throughout. The female gametophyte in gymnosperms is a much more massive structure, in

general, than in angiosperms, and the endosperm is sufficiently developed prior to fertilization of the egg so that it can function immediately and directly in nourishing the embryo. Furthermore at the time of fertilization in many gymnosperms the integumentary tissue has already attained full size. The female gametophyte of most angiosperms, on the other hand, is a simple typically eight-nucleate structure, embedded deeply in maternal tissues which not only are low in reserve foods but are destined to grow considerably after fertilization. The nutrient materials to support this growth must be moved in from other parts of the plant and partitioned between the maternal and filial tissues. The angiosperm endosperm and embryo, therefore, are required to establish themselves in competition with the initially much larger and actively expanding integuments. It is assumed that normal development of the seed hinges at the outset on the capacity of the endosperm to establish and maintain a dominant physiological position relative to the growing maternal tissue surrounding it. Otherwise the partition of nutrients within the seed gets out of balance, the maternal portions of the seed become hyperplastic, the endosperm and embryo are starved, and the seed collapses. Under the delicately poised condition of the juvenile seed a mechanism is demanded which will tip the balance in favor of the endosperm. The secondary fertilization is a method of conferring on the tissue the required advantage.

There are several well established facts in the literature on embryology (*cf.* 217) which point to the endosperm as playing the leading rôle in the young seed. The first of these is the precocious development of the tissue. The endosperm increases with great rapidity both in volume and in number of nuclei or cells immediately after fertilization. Contact with the enclosing maternal tissues is quickly established over a relatively large area. The embryo, on the other hand, develops slowly at first, forming a compact body of cells often projecting into the endosperm at a point opposite the micropyle. On a system of coordinates showing size of tissue as time elapses beyond fertilization the endosperm would be represented by a curve which rises rapidly at the start and attains a maximum early. The embryo lags during this period and enters its major growth phase later.

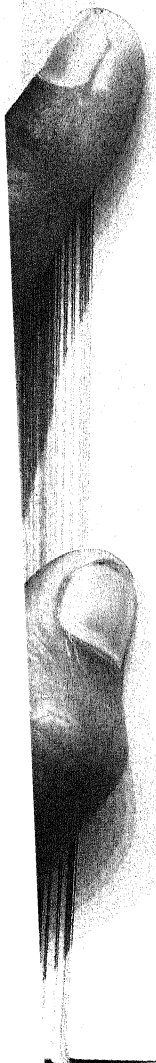
The varied forms which the endosperm takes throughout the

angiosperms and the differentiation of the tissue, including the formation of haustoria, are intelligible in terms of the nutritive function which the tissue serves. Food materials are usually received through the chalaza. The cells of this region of the endosperm tend to be densely cytoplasmic and elongated in the direction of the nutrient stream. In species in which the tissue membranes in the micropylar region are not cuticularized, the greater density of the endosperm cytoplasm in this region is evidence of a secondary source of food uptake. The vicinity of the embryo is likewise one of great metabolic activity, a fact which is reflected in endosperm cells of smaller size and denser cytoplasm.

The Podostemaceae and Orchidaceae lack functional endosperms. It is significant that in these families the embryos are poorly developed and that special structures such as pseudo-female gametophytes and suspensor haustoria are often present, tending to serve the nutrient function of the endosperm.

The assumption that seed collapse following certain types of matings originates in impaired endosperm growth is valid only if it can be shown that development is a function of the hereditary organization of the tissue and that genetic differences may express themselves previous to the onset of breakdown. It has been recognized since xenia in maize was interpreted in Mendelian terms that endosperm characters in the mature seed followed the usual rules of inheritance. Critical evidence was lacking, however, concerning the extent to which endosperm behavior in the very young seed was independent of the maternal genotype. That gap has now been closed. Some workers (*e.g.*, 15, 130, 207) have described conspicuous departures from the normal course of early endosperm development in cereal species crosses, and two of them (19) have shown that at nine stages from zygote to four-celled true embryo in *Medicago sativa* the number of endosperm nuclei following cross-fertilization is larger than after self-fertilization. It can no longer be doubted, therefore, that the young endosperm responds promptly to changes in genetic composition.

The somatoplastic sterility hypothesis formulated by the reviewers in 1940 to account for the high frequency of collapsing seeds following enforced self-fertilization in *Medicago sativa* and later extended by them to *Nicotiana* species crosses and matings between races of tomato differing in degree of polyploidy is founded



on the considerations which have been discussed above. Histological examination of seeds destined to collapse showed that the changes in development eventually terminating in breakdown of the seed originated in the actively growing endosperm soon after fertilization and before the embryo comprised more than a few cells. A general characteristic of the endosperms in seeds unable to continue development was a subnormal growth, as measured by number of nuclei or cells present. The significance of the reduced rate of endosperm development became apparent when it was observed that the conducting tissue leading to the chalazal pocket in these seeds did not differentiate normally and the integumentary tissue, beginning at a point opposite the end of the vascular bundle, became hyperplastic. The first signs of breakdown in the endosperm appeared in the region adjacent to the over-stimulated maternal tissue. Only after definite impairment of the endosperm became visible was it possible to detect an adverse effect on the embryo. It was reasoned that the primary factor in the seed collapse was weak growth of the endosperm consequent upon an unfavorable genetic organization. The immediate effect of the endosperm hypofunction was assumed to be a maldistribution of the nutrients moving into the seed, with the result that the endosperm received less and the integument more than a normal share. Overgrowth of the integument ensued as a consequence of the excessive food supply to that structure. Overgrowth of the integument, in turn, reacted to further depress endosperm development.

Application of the term "somatoplastic" to this form of sterility is open to criticism on the grounds that it overemphasizes a secondary effect—hyperplasia of the adjacent maternal tissue—rather than what was recognized from the start as the primary factor in the phenomenon, namely, subnormal endosperm growth. The term "embryo lethality", as used by Fagerlind (74), is subject to the same objection, that death of the embryo is an effect rather than the cause of breakdown. Furthermore the investigations on seed collapse in the cereals have failed to disclose overgrowth of maternal tissue, although the evidence is clear that, as in the other cases analyzed, endosperm growth is profoundly disturbed. An expression is needed which gives effect to the basic relation of the endosperm to the phenomenon.

The investigations (212) on interspecific crosses in *Datura* and

$4n \times 2n$ and $2n \times 4n$ matings in *D. stramonium* did not lead to a definite conclusion concerning the sequence of changes terminating in seed collapse. A considerable number of seeds were observed in which breakdown appeared to be initiated by the endosperm, but the investigators suggest that since the endosperm cells are larger than those of the embryo, changes in the former are more readily detected than in the latter. In other seeds examined disintegration appeared to start in the proembryo. The initiation of overgrowth of maternal tissue in the collapsing *Datura* seed appeared not to be localized in the vicinity of the apex of the vascular bundle but sometimes occurred in the micropylar region as well. The ease with which the sequence of events leading to breakdown of the seed can be determined is related to the speed with which the degenerative changes proceed. It may be that *Datura* is less favorable material in this respect than some other plants in the Solanaceae. It seems unlikely, in any case, that the disparity in behavior reported by the reviewers for *Nicotiana* and *Lycopersicon*, on the one hand, and for *Datura*, on the other, has any basis other than the difficulty of discerning the facts.

The death of hybrid seeds was attributed to the unfavorable environment afforded by the maternal parent (135, 136): "Such seeds may be viable but their substratum, the mother plant, may not be qualified to afford them normal development". The author believed that his success in rearing the hybrid *Linum perenne* \times *L. austriacum* by artificial cultural methods was due to removal of the embryo from the seed in time to avoid "the fatal influence of the mother". There is little in the subsequent work on hybrid seed failure which supports this point of view.

Kostoff (132) considered that the failure of certain interspecific hybrid seeds in *Nicotiana* to develop to a germinable condition was due to an immunity reaction between the genotypically diverse endosperm and adjacent maternal tissues. The deposition of materials at the boundary between endosperm and endothelium in collapsing seeds was cited as evidence of antigen-antibody activity. Difficulties are encountered in applying this explanation to other cases. The type of seed failure associated with enforced self-fertilization in *Medicago*, for example, is essentially like that occurring in various interspecific crosses (19). It would be necessary on Kostoff's hypothesis to assume in this case that an immunity

reaction developed between tissue elements having their origin in the same individual. The same criticism applies in matings between diploids and their respective autotetraploids.

Failure of the seed to develop normally, in the opinion of Fagerlind (74), is due to disturbance of the equilibrium between embryo, endosperm and maternal tissue. Species, genera and even families are assumed to differ in their sensitivity to an unbalance between these structures. The same tissues are not necessarily always involved. Sometimes the relation between endosperm and embryo may be unbalanced; in others, the disturbance may affect embryo and maternal tissue. Fagerlind is inclined to attribute to the embryo the leading rôle in seed development following species crosses in *Rosa*, on the grounds that the ripe seed is non-endospermic. It is suggested also that disturbance of the equilibrium between embryo, endosperm and maternal tissue may be a secondary effect of the stimulating influence on these tissues of the pollen tube and its nuclei.

The mechanism of seed collapse in the Gramineae has been reserved for separate consideration because of the widespread occurrence following species crosses in this family of irregular mitotic behavior in the endosperm (15, 40, 130). A few cases of endosperm nuclei of abnormal size, shape or chromosome number in aborting seeds have been reported from three other families. Renner (207) observed considerably enlarged nuclei in defective *Oenothera* seeds. Fusion of endosperm nuclei was seen (41) in eight-day-old seeds from the mating *Lycopersicon pimpinellifolium* \times *L. peruvianum*. In neither of these instances, however, are the nuclear irregularities clearly comparable to those in the cereals. Fagerlind (72), on the other hand, has described variations in chromosome number in dividing endosperm nuclei and irregular wall formation at seven days in $2n \times 4n$ and $4n \times 6n$ *Galium Mollugo* seeds which appear to be very similar to those commonly occurring in Gramineae species crosses.

Kihara and Nishiyama (130) observed highly irregular nuclear behavior in the endosperm of *Avena fatua* \times *A. strigosa* seeds, leading to breakdown of the tissue and eventual collapse of the entire caryopsis. Aside from reduction in size the embryos developed fairly regularly during the life of the seed, and the antipodals were reported to degenerate in the usual way at about 72

hours. The observations of these investigators on the "regeneration" in some *A. fatua* \times *A. strigosa* seeds of a normal appearing endosperm tissue from cells adjacent to the antipodals is of possible significance in relation to the problem of irregular mitosis in other parts of the structure. Noting that the cytoplasm of the free-nucleate endosperm in 48-hour seeds was denser in the vicinity of the antipodals than in other parts of the tissue, Kihara and Nishiyama concluded that this was a region of more adequate nutrition and that these better nourished cells eventually proliferated in some seeds and displaced the disorganized tissue in the other parts of the endosperm.

Brink and Cooper (21) rejected the idea that the mitotic abnormalities in the endosperm of *Hordeum jubatum* \times *Secale cereale* are a direct result of the hybrid condition of the nucleus. The grounds for this conclusion were that (a) the embryo which is also a hybrid structure shows regular mitosis, and (b) the antipodal cells, which carry a haploid complement of *H. jubatum* chromosomes only, also may divide irregularly after the hybrid fertilization. It appeared more likely that the mitotic abnormalities in the cross-bred endosperm were of secondary origin and that an explanation of the phenomenon should be sought for in the conditions underlying early growth of the hybrid tissue. The antipodals form a massive tissue in the female gametophyte of the Gramineae and, moreover, persist throughout the nuclear stage of endosperm development. Since they lie athwart the nutrient stream and normally enlarge greatly at fertilization, they might be a factor in the differential behavior of hybrid and normal endosperms.

The weak response of the antipodals to the advent of *Secale* sperm to the embryo sac of *Hordeum jubatum* relative to the pronounced activation of this tissue immediately following normal fertilization has been recounted earlier in this review. It was postulated by the writers that the failure of rye sperm to stimulate the antipodals to their normal secretory activity resulted in undernourishment of the endosperm mother cell and its immediate descendants. Since division of the primary endosperm nucleus is not delayed, or only slightly delayed, by the hybrid fertilization, the food demands of the young endosperm are not diminished. There is no direct evidence concerning the substances which are in short supply, but, since mitosis is upset, it is not improbable that

an essential constituent of the nucleus is involved. Although the seed may live beyond the antipodal stage, recovery of the hybrid endosperm is impossible because irregular assortment of the chromosomes is an irreversible change.

The writers have expressed the view that the abnormal mitotic behavior in the endosperm of barley \times rye and similar hybrids in the Gramineae is a special aspect of seed failure associated with the intercalation between endosperm and host plant of an active antipodal (gametophytic) tissue. To what extent nuclear disturbances in the endosperm following wide crosses is coextensive with the occurrence of large and persistent antipodals is not known. One apparently parallel instance outside the Gramineae has been described in *Galium Mollugo* (72). A well developed antipodal apparatus continues to function after fertilization in this species, as in several other members of the family Rubiaceae.

The possibility is to be borne in mind in this class of cases that even if nuclear division were not upset, the endosperm might still be incapable of supporting the development of functional seeds. The initial post-fertilization stimulus to antipodal activity probably proceeds from the triple fusion nucleus. If the endosperm fails to function in this primary relation, the opportunity for any other inherent defects to express themselves obviously is precluded.

Thompson and Johnston (251) conclude from their studies on seed collapse in the *Hordeum vulgare* \times *Secale cereale* cross that breakdown of the endosperm is due directly to the hybrid nature of this tissue itself, "or to an unfavorable reaction of some kind with the mother plant, resulting from that constitution". They rule out the somatoplastic sterility hypothesis for this and other cases of seed failure in the cereals on the grounds that there is no evidence for overgrowth of the maternal tissues following wide crosses in the Gramineae. The slight difference in behavior of the antipodals which Thompson and Johnston report on substituting rye sperm for barley sperm at fertilization of *Hordeum vulgare* appears to these investigators to be an effect rather than a cause of endosperm breakdown.

It is apparent from the foregoing discussion of the histological data that some of the main issues concerning the mechanism of seed failure can now be defined in terms which afford definite guidance to the investigator. There is disagreement between different work-

ers, however, on several facts which appear to be important for a general interpretation of the phenomenon. An attempt will not be made here to summarize all the controversial points. A few questions, however, may be set down which in the reviewers' judgment should repay further study.

1. Even though the weight of evidence indicates that breakdown of the seed originates in the young endosperm, does the embryo sometimes play a direct rôle also? This is a pertinent question in cases in which plants grown from shrunken, but viable, seeds either die as seedlings or remain weak throughout a longer period of development. Mangelsdorf (159) has presented evidence indicating that certain genes for defective seeds in maize exert a parallel effect on endosperm, embryo and plant. It has been demonstrated that this relation is not true generally, but it may hold in a significant proportion of cases. During early seed development the embryo is a highly dependent nursling of the endosperm. Later, however, embryo growth probably becomes increasingly a function of the genotype of the zygote itself. These considerations led to the suggestion (22) that during the later stages of seed growth, "embryo lethal genes", if such are present, might manifest their effects.

2. Does early fruit development sometimes react unfavorably on endosperm growth by lowering the plane of nutrition of the young seed to a critical level, or by other means? A rapid enlargement of the fruit commonly occurs in the flowering plants directly after syngamy. It is now known that this is also a period of rapid endosperm growth. The two phenomena may be correlated. Tukey (254, 255) and others have shown that, in general, poor seeds form in the early ripening varieties and good seeds in late ripening varieties of *Prunus*, regardless of pollen source. It would be of interest to know whether the poor seeds characteristic of the early varieties are a consequence of a maternally conditioned difference in fruit growth reacting upon the seed during the stage in which the endosperm is forming.

3. The antipodals persist after fertilization throughout a few families and in certain genera and species in others. Does the continued activity of this gametophytic tissue intercalated between the vascular bundle and the endosperm give rise to a special set of conditions of significance for seed development? The evidence on this question from matings resulting in differential seed formation

is limited at present to two cases in the Gramineae. Further study of the problem in both monocotyledons and dicotyledons is called for.

4. Autoparasitism, which Goebel (83) defines as one organ living at the expense of another on the same plant, is a very common phenomenon in fruit and seed development. Evidence for it is seen in the ovule and seed in the absorption of macrospores, synergids, antipodals and endosperm as a cycle of growth in one organ supersedes that in another. Does this mode of nutrition involve a special class of metabolites whose variations in quality and amount are an important factor in seed development?

5. Development of the seed involves the movement of a relatively large volume of nutrients into this structure from other parts of the plant. Brink and Cooper (20) have shown that definite changes in the conducting tissue within the seed occur in response to variations in the genetic organization of the endosperm. The endosperm may also affect the behavior of the conducting tissue leading to the fruit, thus influencing the rate of flow and partition of incoming food materials not only in the seed but also in the tissues associated with it. The possible relation between endosperm activity and formation of an abscission layer is pertinent in this connection.

RELATION OF SEED ABORTION TO OTHER PHENOMENA WITH WHICH IT IS OFTEN ASSOCIATED

Various other direct and indirect effects on reproduction and development, in addition to the formation of defective seeds, may be associated with the types of matings which have been listed earlier as sometimes leading to seed collapse. Some of these effects are not relevant to the present discussion. It is important for an understanding of endosperm relations, however, that consideration be given to pollen tube growth, potential viability of the embryo in the subnormal seed, and fertility of the resulting plant. The apparent relations between these phenomena and impaired seed development may be summarized under three propositions:

1. Pollen tube growth in matings leading to reduced development or abortion of the seed varies widely:

(a) Pollen tube growth is slowed down and frequently arrested, e.g., in self-fertilization of *Secale cereale* (140) and *Medicago sativa*

(19); in matings involving parent races with the same or similar genomes but in different number, particularly when the staminate parent possesses the higher chromosome number, as in $2n \times 4n$ *Datura stramonium* (27); in many interspecific crosses, e.g., *Nicotiana rustica* \times *N. glutinosa* (38).

(b) No effect on pollen tube growth is detectable, e.g., in some monohybrid defective seed types in *Zea mays* (159); in the *Hordeum jubatum* \times *Secale cereale* mating fertilization appears to occur as promptly and as frequently as in *H. jubatum*, normally selfed (40).

(c) Pollen tube growth is faster than in other matings on the same pistillate parent giving normal seeds, and more ovules become fertilized; e.g., pollen tube growth is restricted and many of the seeds formed are plump in $4n \times 4n$ matings, as compared with nearly normal pollen tube growth, high frequency of fertilization and abortive seeds in the $4n \times 2n$ combinations in *Zea* (201) and *Lycopersicon* (41).

Fagerlind (74) has emphasized the statistical fact that many matings which yield shrunken seeds also involve retarded pollen tube growth. The same causal factors may, in the reviewers' judgment, be directly operative in some cases, whereas in others the parallelism appears to be due to a remote common factor or is purely fortuitous. The writers (19) have suggested that the low frequency of fertile ovules resulting from enforced selfing of *Medicago sativa* is a result of self-incompatibility. Presumably the latter is due to the action of particular incompatibility genes affecting pollen tube growth. The high proportion of aborting seeds following this mating, on the other hand, is attributed to an inbreeding depression effect on the endosperm in which many different genes may be concerned. If this view is correct, then the two phenomena are quite distinct. The results on *Pyrus* (265) and *Secale* (140) may be similarly interpreted.

It is true, of course, that the pollen tube is partly, and the endosperm and embryo are wholly, dependent for their nutrition upon the same maternal plant. This circumstance affords a basis for correlated physiological responses. Since the tissues involved and the duration of growth of the pollen tube and the seed are different, however, there is considerable opportunity for independent variation in the two phenomena.

Examples of both independent and correlated variation in pollen tube growth and seed development are known in maize. The sugary (*su₁*) gene, which causes a wrinkling of the endosperm and reduces seed weight about 10%, ordinarily is transmitted through the male gametophyte with normal frequency. Conversely the *Ga* gene markedly retards pollen tube growth in *Ga* stylar tissue, but is not known to influence growth of the seed (61). On the other hand, some of the recessive genes causing defective seeds in maize (148, 159) may possibly have a slightly adverse effect on the male gametophyte. In these cases somewhat fewer than the expected 25% of defective seeds appear on segregating ears.

The waxy (*wx*) gene in maize affects both the male gametophyte and the endosperm in a clear-cut way. The reserve starch laid down in both these structures under the action of the *wx* gene stains reddish-brown with iodine rather than bluish-black as does ordinary starch. Waxy seeds, on the average, are about 3.5% lighter than non-waxy seeds on the same ear. Brink (17) showed that heterozygous waxy plants on selfing form about 24% waxy seeds instead of the proportion expected on Mendelian grounds. It was pointed out that since the deficiency was the same in the upper and lower halves of the ear it must be due to some characteristic of the waxy male gametophytes which was manifested during the period in which the pollen tube was dependent on the reserve foods from the pollen grain. Subsequently Sprague (233) demonstrated that waxy pollen tubes establish themselves in the stylar tissue more slowly than non-waxy pollen tubes.

The sugary (*su₁*) gene, which in *Su₁su₁* plants has no detectable effect on pollen tube growth, nevertheless may influence the behavior of *wx* male gametophytes. Brink and Burnham (18) found that the *su₁* gene exaggerates sharply the deficiency of waxy seeds when *su₁su₁Wxwx*, but not *Su₁su₁Wxwx*, plants are self-pollinated. It is apparent that the action of the sugary gene on waxy pollen tube growth is exerted indirectly through the staminate parent. One may assume that the genes in maize which adversely affect both pollen tube growth and endosperm development condition certain chemical reactions which are common to the two structures. The influence of the gene on the male gametophyte may be post-reductional, as in the waxy, or it may be initiated in the paternal parent, as in the sugary-waxy example just described.

2. The inherent potentiality of the embryo to grow into a seedling and plant varies, to a large degree, independently of the grade of seed development.

Both seed development and plant vigor vary continuously so that any classification must be rather arbitrary. Examples are known, however, which fall into the following categories:

(a) Hybrid vigor is expressed both in the seed and in the resulting plant; *e.g.*, in many crosses between distinct inbred lines of *Zea*. The reduction in seed and plant size regularly found on inbreeding maize is the obverse of this phenomenon.

(b) Outcrossing does not alter seed size, but the offspring show heterosis. This is frequent in varietal crosses and sometimes occurs in interspecific matings (273).

(c) Weight of seed may be reduced one-half to four-fifths, but the resulting hybrid plants show great vegetative vigor; *e.g.*, in tetraploid and hexaploid wheats crossed with *Agropyron glaucum* and *A. elongatum* (5).

(d) Germinable seeds form, but the resulting seedlings and plants develop weakly or die; *e.g.*, simple recessive chlorophyll-deficient types and dwarfs in maize; *Crepis capillaris* \times *C. tectorum* (104); *Melilotus alba* \times *M. dentatus* (229); *Triticum monococcum* \times *T. aegilopoides* (224).

(e) The development of functional seeds fails altogether or is very drastically impaired following crossing, but the hybrids, if they can be grown by special means, are thrifty and sometimes even more vigorous than the parents, *e.g.*, *Linum perenne* \times *L. austriacum* (136), *Hordeum jubatum* \times *Secale cereale* (22).

(f) Seed development is inferior and the resulting plants are weak or inviable: *Triticum dicoccum* var. Khapli \times *T. vulgare* (15), several defective seed types in maize (159).

The lack of correspondence between grade of seed development and capacity for growth of the embryo becomes evident at once on considering the results of certain reciprocal interspecific crosses. The Gramineae afford several clear-cut examples. Thompson (247) has reported, for example, that the matings *Triticum vulgare* \times *T. dicoccum* and *T. vulgare* \times *T. durum* yield seeds which, although smaller than those of normal *T. vulgare*, are plump and healthy. The seeds from the respective reciprocal crosses, on the other hand, are wrinkled and germinate poorly. The genetic con-

stitution of the embryos in each pair of reciprocals is the same, and the plants to which they gave rise look and breed alike. Obviously when reciprocals vary in grade of seed development, the difference is quite unrelated to the inherent developmental capacity of the embryos.

The class of cases falling under (e), above, is of particular interest for the present discussion in that breakdown of the seed during development is usually complete. In spite, however, of the failure of seeds of this kind to attain a germinable condition, the embryo may be potentially viable and capable of producing a vigorous plant. Laibach (135, 136) was the first to point out that death of the seed following an interspecific cross was not proof that the enclosed embryo was inherently inviable. The basis for this conclusion was Laibach's demonstration that the embryos in the abortive seeds formed on crossing *Linum perenne* with *L. austriacum* could be dissected out and successfully reared on an artificial medium.

The hybrid between *Hordeum jubatum* and *Secale cereale* (22) is also instructive in this connection. As described elsewhere in this review, histological study revealed that seeds resulting from this mating fail relatively early in development, due to a complete breakdown of the endosperm. The embryo, which was observed to differentiate normally as long as the seed remained alive, was shown to be capable of giving rise to a vegetatively normal plant if excised from the immature seed and reared to the seedling stage on an artificial medium. The rather distant relationship which has been assumed to exist between the parent species was borne out by the limited pairing of chromosomes observed in the hybrid. The findings in the *Hordeum* \times *Secale* mating make it clear that collapse of the embryo within the seed is not analogous to the death from inherent causes of a hybrid seedling, the essential difference being merely that "lethal" genes become active at an earlier stage in the ontogeny of the young sporophyte. The arrested growth of the embryo is rather a secondary effect of endosperm disfunction.

The evidence from maize is interesting in relation to the question whether genes having pronounced effects on seed development are limited in their action to that stage of development or exert general effects on the growing plant as well. Mangelsdorf (159) found that genetically defective seeds in maize were distinctly inferior to

normal seeds harvested at dates sufficiently early to make them comparable in dry weight both in germination and growth of the resulting plants. With few exceptions the defectives which germinated died as seedlings. Homozygotes were reared to maturity, however, in four independent lines. The plants remained weak and unthrifty throughout development and produced little pollen and few ears. Mangelsdorf concluded that the deleterious influence of the defective genes was not limited to the endosperm and embryo but was expressed at all stages of development which the sporophyte attained.

Another defective seed type in maize, mentioned in an earlier section as being under study by the present writers, appears to have a rather specific effect on seed development. Weight of the mature caryopsis of this defective, on the average, is only about 25% of normal, germination is low, and most seedlings die. The survivors, however, are capable of developing into large vigorous plants which produce an abundance of pollen and seed. Even in this case, however, size of the adult plants grown from defective seeds is significantly less than that of normal sibs. It is not known at present whether the reduced stature is a consequence of the pronounced weakness at the seedling stage or is due to a continuing but much abated action of the defective seed gene on plant growth.

3. The grade of seed development following a species cross is not closely associated with the fertility of the resulting hybrid plant.

Many factors, including gene homology, structural relations of the apposed chromosome sets and degree of polyploidy, condition the fertility of an interspecific hybrid plant. The relation of polyploidy to seed development is considered under another heading. So few data concerning the effects on seed growth of cytogenetic factors other than polyploidy are available that a discussion of them in relation to fertility is scarcely practicable. Attention may be called, however, to some bits of evidence pointing to the validity of the general statement above.

No species crosses have been described giving shrunken seeds from which fully fertile offspring may be grown. If the parents are sufficiently unlike so that they form poor seeds on crossing, apparently the fertility of the resulting hybrid is always reduced in some degree. (The various recessive genes in *Zea mays* which adversely affect seed size, however, are not known to cause any

meiotic irregularities.) But the two phenomena are not closely correlated. This is attested by the fact that among the hybrids which regularly die in the seed but which have been reared with the aid of artificial cultural media, there are some which are at least moderately fertile. Laibach (136), for example, reared by artificial means a hybrid from one of 27 severely shrunken seeds obtained from the mating *Ladanum pyrenaica* \times *L. ochroleuca*. The plant, although weaker than the parents, yielded about 100 normal appearing seeds on selfing. The embryo formed from the cross *Lilium Henryi* \times *L. regale* ordinarily dies in the seed. Skirm (226), however, succeeded in rearing this hybrid by cultivating excised embryos on an artificial medium, and found it partially fertile on backcrossing to each of the parent species. Seedlings from the backcrossed seeds were obtained by ordinary methods. Thus some interspecific hybrids lying so far down on the scale of seed quality that special measures are required to rear them have proved to be inherently capable of sexual reproduction. Armstrong (6) found little relation between the readiness with which certain intergeneric hybrids in the Gramineae may be obtained and their fertility. He crossed a series of varieties of *Triticum vulgare*, *T. durum*, *T. dicoccum*, *T. turgidum* and *T. timopheevi*, used as the pistillate parents, with *Agropyron elongatum* and *A. glaucum*, respectively. The matings with *A. glaucum* gave the higher percentages of florets setting seed except in the combinations with *T. dicoccum*. All the *A. glaucum* F₁ hybrids reared, however, were sterile. The corresponding *A. elongatum* hybrids, on the other hand, were partially fertile.

It is apparent from the above discussion that the relations between seed development, on the one hand, and pollen tube growth, potential viability of the embryo and fertility of the resulting plant, on the other, cannot be summarized in simple terms. One conclusion, however, emerges. The seed is the site of phenomena which, to a very significant degree, vary independently of those associated with pollen tube behavior and of the inherent capacity for growth and fertility of the ensuing sporophyte.

XENIA

Focke in 1881 applied the term "xenia" to all deviations from the normal in form or color of any part of the plant attributable to the

action of foreign pollen. Changes resulting from a reduced number of seeds alone were excepted. This very broad definition, however, is no longer followed. Xenia, as currently employed, refers to the immediate effect of foreign pollen on visible characters of the endosperm. Direct action of the pollen on the embryo, clear-cut examples of which have become well known through Mendel's work on the garden pea, is excluded on the grounds that the genetic characteristics of the embryo are unique only in being expressed at an early stage in ontogeny of the sporophyte. Effects of differential pollination on the maternal tissues fall in a different category and have been termed "metaxenia".

Zea mays affords the classical examples of xenia. Webber (270) has reviewed the early work on this plant. Direct effects of pollinating sugary maize with non-sugary varieties and of mating white-seeded strains with those having pigment in the aleurone layer or throughout the endosperm were repeatedly observed by early investigators, but conclusive evidence regarding the basis of xenia was not forthcoming from their work. An explanation of the phenomenon awaited further knowledge of the fertilization process in flowering plants and an understanding of the Mendelian mechanism of inheritance.

The discovery of double fertilization by Nawaschin (185) and Guignard (86) provided a basis for the interpretation of xenia. De Vries (264) pollinated sugary maize, which has wrinkled kernels, with pollen from a non-sugary strain. He observed, as had others before him, that all the seeds resulting from this cross were smooth, like those of the staminate parent. De Vries proved by controlled matings that the parental strains bred true for their respective kernel types. He concluded, in the light of Nawaschin's and Guignard's cytological findings, that when a direct influence of the pollen occurs the change is due to hybridity of the endosperm. Correns (43), whose experiments with maize were concurrent with and similar to those of de Vries, had come to the conclusion before double fertilization was discovered that either the secondary nucleus of the embryo sac fuses with one of the sperms from the pollen tube or that xenia is due to an enzymatic influence of the hybrid embryo. He pointed out in a paper, which appeared shortly after that of de Vries, that the actual discovery of double fertilization established the correctness of the former view.

It is of interest to note in passing that the observations on maize which de Vries and Correns related to the problem of xenia in 1899 were among the facts which led these investigators to announce the rediscovery of Mendel's laws a year later.

Since 1900 several additional examples of xenia in maize have come to light. The evidence bearing upon them may be found in a comprehensive summary of maize genetics (69). The characters displaying xenia are inherited in accordance with the ordinary Mendelian rules, applying to attributes manifested in the sporophyte. Xenia has been found to arise under two sets of conditions: (a) in monohybrid endosperms in which the dominant member of the allelic pair is derived from the staminate parent, and (b) in endosperms in which dominant genes with complementary effects are brought together by crossing.

Any suspicion which may have remained that xenia is due to an influence on the endosperm of a hybrid embryo was dissipated by Sprague's (232) work on heterofertilization. Sprague was able to show, by use of pollen mixtures from particular strains of maize carrying genes having differential color effects on both endosperm and embryo, that the two fertilizations occurring within a female gametophyte occasionally involve participation of sperms arising from different pollen grains. The gene *R* is essential to formation of color in the endosperm and embryo. Among 719 seeds resulting from matings of the type $rr \text{ } \text{♀} \times (RR + rr) \text{ } \text{♂}$, for example, 325 had colored (*R*) and 394 colorless (*r*) endosperms. Ninety-nine seeds in the latter class, however, possessed colored embryos. In these exceptional seeds the male nuclei involved in fertilization of the central cells carried the gene *r*, thus giving unpigmented endosperms, whereas those conjugating with the eggs were *R*, producing pigmented embryos. The fact that the endosperms in these 99 seeds remained colorless in spite of their association with colored embryos shows that the phenotype of the former tissue is independent of the constitution of the embryo.

METAXENIA

Focke's (77) contention that cross-fertilization as compared with self-fertilization may differentially affect not only the endosperm and embryo but certain tissues of the maternal plant, particularly the fruit, has been substantiated by later investigators. Some of

the cases which Focke and his contemporaries cited as examples have not been confirmed; but there is no longer any doubt about the validity of the claim that, in addition to influencing the appearance of embryo and endosperm, differential matings may modify fruit development also. Swingle (245) has proposed that the latter phenomenon be termed "metaxenia".

A striking case of metaxenia in the date palm, *Phoenix dactylifera*, has been described (190, 245). The Deglet Noor variety when crossed with two other strains, Mosque and Fard No. 4, gives fruits differing markedly in size and time of maturity in the two cases. The Mosque mating yields fruits and seeds heavier by 9.5% and 58.9%, respectively, than those from the Fard No. 4 pollinations. The absolute differences observed in dry weight were 67.4 g. for the fruit tissue and 40.6 g. for the seeds, per 100 fruits. Types of pollen resulting in smaller fruits, in general, induce early ripening. The mating *P. dactylifera* \times *P. canariensis* is an exception to this rule in that it results in small very late ripening fruits. The seeds produced in this combination are abnormally tapered at the base. Nixon reports also that seeds from the *P. dactylifera* \times *P. Roebelenii* cross are characterized by a peculiar dorsal depression which is attributed to abnormal endosperm development in the vicinity of the embryo.

Several cases of metaxenia have been reported in the apple. The phenomenon is not easily demonstrated in this plant because differences in fruit size associated with variations in seed number tend to obscure it. Lewis and Vincent (146) found that the Yellow Newton \times Jonathan and Yellow Newton \times Grimes Golden crosses gave fruits heavier by 9% and 15%, respectively, than the Yellow Newton \times Spitzenburg mating. The variety Baumann's Reinette was observed (134) to give larger fruits than several other varieties with which it was compared as the pollen parent in crosses. Krumbholz noted that metaxenia tends to be expressed in the apple only when the crop is heavy, that is to say, when competition between fruits for the available food supply is a significant factor. No effect of the pollen parent in the apple was found (59) on shape, acidity and total sugar. Nebel (186) concluded that differences in shape of fruit attributable to metaxenia remained in Famuese \times Yellow Bellflower and Famuese \times McIntosh crosses after allowance was made for the larger number of seeds resulting from the former

mating. Likewise apples from McIntosh pollinated by Astrachan possessed a somewhat lower pH and a definitely higher total acidity than fruits from McIntosh \times Yellow Bellflower matings. Keeping quality of the fruits was found to be affected by pollen source in a comparison which Nebel made between apples from McIntosh \times Wilson Red June and McIntosh \times Boiken crosses. Spoilage after nine months was considerably greater in the latter class of fruits.

Schreiner and Duffield (220) report an interesting example of metaxenia in the oak. The fruits of *Quercus Robur* are comparatively late maturing, relative to those of *Q. alba*. The two species were crossed in the spring of 1941, using *Q. alba* as the pistillate parent. A comparison made on September 23 showed that acorns resulting from the cross varied from brown to light-green, whereas those of *alba* were brown and fully matured. The acorns of the staminate parent, *Q. Robur*, were still light-green and unripe at this date.

Kiesselbach (128) observed that weight of the pericarp in the mature seed of maize is increased after outcrossing of inbred strains. Others (4) report another case of metaxenia in *Zea* involving an effect on the ovary wall. Pericarps peeled from sugary and non-sugary kernels borne by the same ears on 72-day-old plants were found to differ in toughness as measured by resistance to penetration by a needle under standard conditions. Pericarps of non-sugary kernels offered an average resistance to puncture of 75 grams. The value for the sugary kernels was only 54 grams. Since the pericarps from both classes of kernel are of the same genotype, it must be assumed that the physical properties of the tissue are influenced during development by the two different kinds of underlying endosperms.

Length of lint hairs and amount of fuzz in cotton are properties of the seed coat. Harrison (98) has observed that both are influenced by differential pollination. The effect of outcrossing was to change these characteristics in the direction of the type of the stock used as the pollen source. Thus pollen of the short-linted, smooth-seeded Hopi cotton, when used on the long-linted, relatively fuzzy Pima cotton, shortened the lint 7.5% and reduced the fuzz on the seeds. Average seed weight was increased about 4% by the cross. Length of lint in the Durango and Acala varieties, which are

short-linted, was increased 8.2% and 13.5%, respectively, following cross-fertilization with the long-linted Pima cotton. Seed weight in the Durango \times Pima cross was increased about 6%. Pima pollinated by Acala, on the other hand, gave an 8.2% decrease in lint length as compared with Pima \times Pima. No regular relation was found between the increase in seed weight associated with outcrossing, which Harrison concluded was an expression of hybrid vigor and metaxenia. It was suggested that the parallelism between the metaxenial effects and the characters of the pollen parent may be due to secretions initiated by the genes conditioning these characters. Harrison notes, however, that similar relations have not been observed in other cases of metaxenia.

Swingle (245) suggested that metaxenia is the result of hormones secreted by the embryo or endosperm, or both, which diffuse into the maternal tissue of the seed and into the fruit, and there exert specific effects according to the genotype of the male parent of the embryo and endosperm. Since this hypothesis was set forward, considerable evidence has been obtained showing that the developing seed is a comparatively rich source of growth hormones and that these substances are intimately associated with fruit development. Gustafson (90) has reviewed in this journal much of the pertinent literature. Later investigations of interest include a study (8) showing that, whereas the total auxin content of maize kernels is very small at the time of pollination, it increases sharply immediately after pollination, a peak being reached between one and three weeks. Hatcher and Gregory (101) report that the auxin content of winter rye kernels is low at the time of pollination and increases to a maximum about six weeks later. Large amounts of growth hormones are released in the ovary following fertilization in *Nicotiana tabacum* (171). Extracts of maize kernel 10 to 20 days after pollination were highly effective in stimulating parthenocarpic development of tomato fruits (281). The differential fruit development characteristic of metaxenia may be conditioned by growth hormones diffusing from endosperm and embryo. Nebel (186) has pointed out that while the known substances in this class do not in themselves show specificities corresponding to the genetic effects, the growth hormones may be influenced differentially by the male parent in quantity or rate of action.

HYBRID VIGOR IN THE MAIZE ENDOSPERM

Collins and Kempton (34) observed that pollination of one variety of maize by another frequently leads to increased kernel size. By choosing parental strains differing in endosperm color, they were able to distinguish and compare outcrossed and non-outcrossed seeds within the same ears following the use of pollen mixtures. Hybrid seed were found to be from 3% to 21% heavier than seeds of the pistillate variety in a series of matings involving widely diverse types. Since the increase in seed weight occurred irrespective of seed size of the pollen-bearing strain used in the outcross, Collins and Kempton considered it to be a manifestation of the vigor usually expressed also in first generation hybrid corn plants.

The increase in kernel size following outcrossing in maize has since been observed by several investigators. Kiesselbach (128) showed that the greater weight may characterize all parts of the kernel. In a group of 19 inbred strains derived from two varieties, for example, in which gross weight of kernel was increased 10.1% by cross-pollination, the increases in embryo, endosperm and pericarp were 11.8%, 10.4% and 3.2%, respectively. Others (194) also found that outcrossing in maize may increase the weight of the pericarp as well as that of embryo and endosperm.

The hope expressed by Collins and Kempton (34) that the increase in weight of seed resulting directly from cross-pollination might be used to predict grain and forage-yielding capacity of maize hybrids has not been realized. Corn breeders have found that, in general, reciprocal hybrids between inbred strains are indistinguishable at maturity. On the other hand, as others (*e.g.*, 126, 234) have demonstrated, the immediate effects on seed size of reciprocal crosses may differ considerably. No correlation between forage-yielding ability and the increased weight of hybrid over selfed seed on the same ears was detected (194) in an extensive series of comparisons. A doubtfully significant correlation of $r = .39$ appeared between this variable and grain-yielding ability of the hybrid strains.

Griffiee (84) compared seed size in wheat following pollination of castrated flowers with pollen from the same and unrelated strains. Weight of the mature hybrid seed was 24%, 60% and 37% greater than that of the selfed seed in three matings and 7% less in a

fourth combination. Hybrid vigor in the seed has been observed also in rye which is wind-pollinated. The cross-bred green kernels in a yellow kernelled variety of rye grown adjacent to a green sort were heavier than the type (188). The increases, based on many thousand kernels, were 13.6% and 9.6% in two successive years.

Enhanced growth of the endosperm after cross-breeding is a form of xenia. It differs from the classical cases, however, in resting upon complex rather than relatively simple genetic relations.

The physiological basis of hybrid vigor has been little studied. In a comparison of auxin content of 17-day old kernels on four inbred strains of maize after selfing with that of kernels of the same age resulting directly from intercrosses between these strains, the free auxin and auxin precursor concentration in the hybrid kernels was not regularly greater than that in the inbred kernels (8).

ENDOSPERM MOSAICS IN MAIZE

Kernels showing alternative genetic characters in different parts of the endosperm are fairly common in maize. Investigations dealing with them have been reviewed (112) in this journal. Attention has been called by several workers to the high frequency of endosperm, as compared with detectable sporophyte, mosaics. Stadler (235) finds many more deficiencies in the endosperm than in the embryo following application of pollen irradiated with x-rays and ultra-violet light, even though, on the average, the two sperms which initiate development of these respective structures could not have been differentially affected by the treatments. Cytological examination of six-day-old endosperm tissue of maize strains showing high rates of mosaic formation disclosed 2.5% to 13.2% abnormal mitotic divisions (32). The irregularities observed included single bridges, fragments, single bridges and fragments, and double bridges. Clark and Copeland suggest that the large number of atypical nuclear divisions may be associated with the rapidity of the cell cycle in this tissue.

A breakage-fusion-bridge chromosome cycle has been described (151) which could account for many of the cases of mosaic endosperm in maize for which genetic evidence has been secured. The fact that the breakage-fusion-bridge cycle always, or at least usually, is confined to the gametophytic and endosperm tissues of the generation immediately following the initial chromosome break is of par-

ticular significance for this problem. The ends of the broken chromosomes commonly heal in the zygote nucleus so that bridge configurations do not appear in nuclear divisions in sporophytic tissues. Healing of the broken ends in the embryo is permanent, since the characteristic cycle does not reappear in the succeeding gametophytes and endosperm.

The reasons why mosaicism and irregular chromosome behavior are more frequent in the endosperm than in the sporophyte are obscure. There is independent evidence, however, showing that the metabolism of the endosperm and gametophytes in maize differs from that in the other tissues. After Weatherwax's (269) demonstration that the principal reserve material in the endosperm of waxy maize stained reddish-brown with iodine rather than blue, as ordinary starch does, others (23, 60, 149) reported a similar condition in waxy pollen. Subsequently Brink (17) found that the reserve starch in the female gametophyte of waxy plants also stained reddish-brown with iodine. The starch of translocation in homozygous waxy plants, including that in the leaf, stem and embryo, on the other hand, gives the usual dark blue reaction with this reagent.

The more recent investigations show that in its physical and chemical properties waxy starch is very similar to glycogen. According to Hassid (100), ordinary starch comprises two substances, amylose and amylopectin. The former consists of long unbranched chains of about 300 glucose residues, whereas amylopectin is built up of branched chains of approximately 25 glucose units per branch. Waxy starch, like glycogen, is devoid of amylose. The glucose residues in the unbranched chains of amylose are joined only through γ -glucosidic-1,4-linkages. Amylopectin also contains γ -glucosidic-1,6-linkages, which are present at the branching points. Others (42) have summarized the evidence indicating that the branched type of starch molecules appear to be due to the concurrent action of two enzymes, one being phosphorylase which is specific for γ -1,4-glucosidic linkages and the other an enzyme which makes 1,6-glucosidic linkages and thus starts branches which can grow in length through the action of phosphorylase. A variety of branched types of polysaccharides may be formed, depending on the ratio of these two enzymes.

Enzyme differences analogous to those associated with waxy starch may occur in conjunction with other endosperm cell con-

stituents such as nucleoproteins. If this is the case there might well be differences between endosperm and other vegetative tissues in the capacity of the chromosomes to produce exact copies in mitotic division and in ability to heal broken ends when these are present. The tendency for endosperm mosaics to occur with relatively high frequency may have its basis in such a mechanism.

DOUBLE FERTILIZATION AND POLYPLOIDY

The secondary fertilization leading to endosperm formation is a unique characteristic of the angiosperms. Flowering plants, likewise, are the only group of organisms in which polyploidy is known to occur with high frequency. Müntzing (177) suggests that there is a causal connection between the two phenomena. It is pointed out that tetraploids are more or less effectively isolated genetically from the diploid progenitors as a result of the seed abortion which follows hybridization between them. The incompatibility results from the disturbed quantitative relations between embryo, endosperm and maternal tissues and arises coincidentally with doubling of the chromosome number. Polyploidy starts from the formation of unreduced gametes or the occurrence of somatic chromosome doubling. It is preserved, according to Müntzing, as a result of the incompatibility manifested in the seed following crossing between different members of the multiple chromosome series.

UNREDUCED PARTHENOGENESIS

The usual type of apomixis leading to seed formation involves parthenogenetic development of an egg produced by an unreduced female gametophyte. The latter arises either because meiosis fails at sporogenesis (diplospory and generative apospory) or directly from a neighboring vegetative cell (somatic apospory). Although the origin and functional relationships of the endosperm have not been critically studied in many of the reported cases of apomixis, some significant evidence is available:

1. Pseudogamy. Fertilization of the endosperm mother cell is necessary for seed production; development of the diploid egg is parthenogenetic and may be autonomous. Formation of the endosperm is initiated by union of a male nucleus with the central nucleus which consists of the two polar nuclei in a fused ($4x$) or unfused ($2x$) state. Embryo development may or may not have begun by the time the secondary fertilization occurs.

The real nature of pseudogamy was not understood prior to the work of Noack (191). Focke (77), who introduced the term, supposed that pseudogamy involved formation of an embryo without fertilization in response to foreign pollen. The idea long held sway that embryo and endosperm were stimulated to develop by some special substance of pollen tube origin acting on the female gametophyte (147, 279).

This early view was brought into question by Pace's (193) work on pseudogamic seed development in *Atamosco texana*. She found that haploid pollen and diploid female gametophytes are formed. One haploid sperm nucleus fuses with two diploid polar nuclei to produce a pentaploid endosperm. The other sperm nucleus enters the egg but fails to fuse with its nucleus. The embryo developing from such an egg is diploid. Much the same type of embryo and endosperm formation was described (169) for *Allium odorum*, a facultative apomict. The investigator pointed out that in *A. odorum* the endosperm is essential for continued development of ovule and ovary. He concluded not only that the endosperm has trophic properties of importance for the female gametophyte but that it also plays an indispensable organizational rôle in seed and fruit formation. The importance of these findings was not recognized prior to the recent investigations on pseudogamy.

Noack (191) described a parallel condition in *Hypericum perforatum* ($2x = 32$), a tetraploid species, which is for the most part aposporous and pseudogamous. The pollen has the reduced number of chromosomes ($x = 16$). Reduced female gametophytes are formed but they usually degenerate early. Very rarely is such a gametophyte functional. Shortly after or during disintegration of the x -gametophyte, an aposporous one with the unreduced number of chromosomes is formed. Pollination is necessary for seed development. Parthenogenetic development of the unreduced egg occurs in about 73% of the ovules following self-pollination. Fertilization of the central nucleus takes place in all cases, and where it was possible to make chromosome counts the number was found to be 80 ($32 + 32 + 16$). Parthenogenetic development of the egg likewise occurs in the cross *H. perforatum* \times *H. quadrangulatum* ($n = 8$). Here again fertilization takes place in the endosperm mother cell. Noack found 72 chromosomes ($32 + 32 + 8$) on the equatorial plates of dividing endosperm nuclei. He concluded that

fertilization of the endosperm is prerequisite for the parthenogenetic development of the egg.

A number of *Potentilla* species are apomictic (173, 176, 179, 180). Pollination is necessary for seed development but the eggs are not fertilized, with the result that the offspring are strictly maternal and completely uniform. According to detailed studies (82) of the early stages in seed development in two pentaploid ($2x = 35$) and two hexaploid ($2x = 42$) biotypes of *P. collina* and in *P. argentia* ($2x = 42$), aposporous female gametophytes are formed in *P. collina* c-b, a pentaploid biotype which is male sterile. Parthenogenesis is autonomous and the embryos become "fairly well sized", i.e., they are composed of a hundred or more cells, but seeds cannot develop, since the endosperm does not develop in the absence of pollination. The other pentaploid biotype (c-g) is not completely male sterile and produces on the average 9% "good" pollen. Apomictic development of the unreduced egg occurs with or without pollination, but fertilization of the central nucleus is required to initiate endosperm and subsequent seed development. The number of chromosomes on the equatorial plates of dividing endosperm nuclei is proof of this fact. Four counts were made and in each case the number approximated that which was to be expected, namely, $\pm 88 \left(35 + 35 + \frac{35}{2} \right)$.

The two hexaploid biotypes c-c and c-d propagate by seeds containing parthenogenetic embryos and endosperms which have probably arisen as a result of fertilization of the central nucleus, since no seeds are produced in the absence of pollination. *P. argentia* is likewise a hexaploid in which pseudogamy occurs. Three equatorial plate stages in dividing endosperm cells from different ovules were found whereon the chromosomes could be counted, and in each case the number was approximately 105 ($42 + 42 + 21$). In the light of these facts the authors conclude that pseudogamy consists of two processes, parthenogenetic development of the egg and formation of the endosperm following fertilization of the central nucleus.

Detailed studies of seed development in apomictic strains of *Poa alpina* (94, 95) and *P. pratensis* (2, 189) likewise reveal the occurrence of pseudogamy in these species. Development of the egg is autonomous, whereas pollination and fertilization of the endo-

sperm mother cell are necessary for seed development. Håkansson has described the fusion of one male gamete nucleus with the two polar nuclei to produce a pentaploid endosperm in three apomictic forms of *P. alpina*. Endosperms are not formed unless fertilization of the polar nuclei occurs. Aposporous female gametophytes develop in the apomictic strains of *P. pratensis* ($2x = 72$). Both Åkerberg and Nielsen found approximately 195 chromosomes, the $5x$ number, in dividing endosperm cells, indicating that fertilization of the central nucleus likewise takes place in these apomictic strains. Pollination is necessary for seed formation in the apomictic races of both species.

Pseudogamy, in all probability, occurs in various species in which the initiation of both embryo and endosperm formation heretofore has been considered autonomous. A number of forms are known where pollination is necessary for seed development, but no detailed studies of the early stages have been made. A large number of apomicts are known in the Rosaceae (239). The pollen is regularly formed and completely fertile in *Alchemilla* (14, 82, 181). An aposporous female gametophyte is formed. Pollination is necessary for embryo and endosperm development. The pollen tubes grow down through the stigma and style to the ovule. Böös suggested that a hormonal stimulation of the egg and central nucleus occurred as a result of the presence of the pollen tube. Although actual fertilization of the endosperm mother cell has not been observed and no cytological study of the endosperm has been made, the authors (82) conclude that the apomictic seed production in this species is probably pseudogamous, since pollen tubes are always present when the embryo and endosperm begin development. A similar conclusion might well be drawn for certain species of *Rubus* and *Rosa*. A series of pseudogamic apomicts are known in the subgenus *Eubatus* of *Rubus* (88, 127, 147) and in the genus *Rosa* (e.g., 89, 246). These apomicts are all polyploids ranging from triploids to octoploids. Pollination is a prerequisite to seed formation, and following wide crosses matroclinous offspring are regularly produced.

Both haploid and diploid female gametophytes develop in *Oxyria digyna* ($n = 7$) (67). A pollen tube enters the micropylar region of the gametophyte and penetrates one of the synergids. Shortly thereafter the male gametes appear to disintegrate and fertilization

does not take place. Edman came to the conclusion that the haploid eggs are sterile and only diploid eggs develop further. Whether pollination is necessary for the stimulation of embryo development was not determined, and no report was made as to the number of chromosomes in the nuclei of the endosperm. Later (68) he examined early stages of seed formation in *Atraphaxis frutescens* ($2x = 45$). Some functional pollen is formed by this species, and pollen tubes could be seen in the micropylar regions of female gametophytes wherein embryos and endosperms are beginning to develop.

2. Autonomous apomixis. Both the unreduced egg and the central nucleus undergo a series of divisions to develop embryo and endosperm, respectively, without fertilization. The polar nuclei may either fuse prior to further development of the endosperm mother cell, in which case the chromosome number of this tissue is $4x$ (1), or they may divide without fusing, the endosperm nuclei then having the same chromosome complement as those of the embryo and maternal tissue (217, 218). Pollination is not a prerequisite for seed development.

The two polar nuclei fuse very early in *Erigeron annuus* = *E. ramosus* ($2x = 27$) and in *E. strigosus* (105, 152, 230). This fused nucleus undergoes a series of divisions to form a multinucleate endosperm prior to the division of the egg. No pollination is necessary, since decapitated buds produce seeds. Holmgren (105) also found that the polar nuclei may fuse prior to endosperm development in *Eupatorium glandulosum*, and a cellular endosperm develops. In some instances he noted that the polar nuclei divide once to give a four-nucleate endosperm. Before further development ensues two or more of these nuclei fuse, and thereafter a cellular endosperm is produced. Dermen (62) likewise noted a fusion of the polar nuclei in *Malus hupehensis* ($2x = 51$) prior to endosperm formation. *Taraxacum officinalis* ($2x = 24$) and *Hieracium aurantiacum* ($2x = 36$) are apomictic species. Since Stork's (240) repetition of the castration experiments of Runkiaer it has been recognized that the common American dandelion, which is triploid, regularly forms seeds in the absence of pollination. The writers have examined material of these two species and find ± 48 and ± 72 chromosomes, respectively, on the equatorial plates of dividing endosperm nuclei, which definitely indicates a fusion of the polar nuclei.

Development of the endosperm without prior fusion of the polar nuclei has been described as taking place in some apomictic species (82, 217, 236). The polar nuclei behave as separate entities so that a $2x$ -endosperm is formed. Juel (120) reported this type of development for the seeds of *Antennaria alpina*. The polar nuclei are closely appressed to each other in the mature gametophyte. They then separate and each enlarges and undergoes a series of mitoses. The egg divides shortly after initiation of the endosperm. A similar type of endosperm development has been described for seven other species of *Antennaria* (236). An examination of both Juel's and Stebbins' figures reveals the possibility of another interpretation. Each of the closely adjacent polar nuclei in *A. alpina* (120) has two nucleoli, whereas the two enlarged nuclei are multinucleolate. Their increased size and multinucleolate condition would indicate that these nuclei have probably arisen as the result of division of the fused polar nuclei. A similar multinucleolate condition is present in the endosperm nuclei of *A. petaloides* (236). The number and size of the nucleoli at late telophase have been shown to be useful criteria for judging the degree of polyploidy of a nucleus (10, 170). The endosperm in *Calycanthus* (222) appears to develop from an endosperm mother cell in which the upper or micropylar nucleus alone functions.

Some *Alchimella* biotypes, subgenus *Eualchimella*, have very high chromosome numbers. Eight such forms—not including *A. speciosa*—showed little or no pollen (82). In the late bud stages mature female gametophytes are present, and in some instances the eggs are dividing or have divided to form two-celled proembryos. The associated endosperms contain two to eight nuclei. Because of this early development of the endosperm, they conclude that "fusion of the polar nuclei is most likely omitted".

Adventitious Embryony

Adventitious embryony, wherein one or more adjacent maternal cells become meristematic and grow into the female gametophyte or endosperm to form embryos, is known to occur in many species (217, 271). Such development may be initiated after fertilization has taken place, as in *Citrus*, or without the stimulation of pollination, as in *Euphorbia*. Normal double fertilization occurs in *Citrus* (79), but the embryo developing from the fertilized egg is in-

herently weak. It grows much slower than the adventitious embryos and may even degenerate in the course of seed development so that matroclinous offspring are, for the most part, produced. Endosperm formation following fertilization of the central nucleus is a prerequisite for the development of the seed. On the other hand, the endosperm develops without fertilization in *Euphorbia dulcis* (29). The egg degenerates and the embryo is of nucellar origin.

It can be seen from the foregoing discussion that the nature of the endosperm in apomicts and the course of development of this tissue need careful study in order to understand seed formation in these species. The early stages in seed development in many of the species wherein apomixis is known to occur (217, 237) merit re-investigation in order to determine whether pseudogamy occurs, and, if not, whether the two polar nuclei fuse prior to endosperm development. This can best be accomplished by examining the stages leading up to initiation of the endosperm and by computing the number of chromosomes characteristic of this tissue in the various species.

Attention has been called (237) to the advantages which apomicts afford for the study of various problems of major botanical interest such as the nature of meiosis, the alternation of generations, species differentiation and plant distribution. One may add that further investigation of plants of this class also may shed additional light on the unique secondary fertilization in which the endosperm of angiosperms normally takes its origin. The recent findings on pseudogamous forms show that fertilization of the central cell is essential to seed development. Seed is produced in autonomous apomicts, on the other hand, without fertilization of either the egg or polar nuclei. The conditions which permit development of the seed to maturity in the latter class in the absence of the secondary fertilization remain to be discovered.

REDUCED PARTHENOGENESIS AND APOGAMETY

Haploid sporophytes have been found in experimental cultures of various angiosperms. Darlington (58) has tabulated 36 cases. These haploids arise, presumably, from unfertilized eggs (reduced parthenogenesis) or from other cells carrying the x number of chromosomes (apogamety) in normal female gametophytes. Sev-

eral have occurred as haploid-diploid twins (271). Twin embryos, one arising from the zygote and the other from a synergid, have been observed (37) in about 1% of the young seeds in seven species of *Lilium*. Since haploids are rare and are not usually recognized before the seedling stage, at the earliest, very few data are available concerning the endosperms associated with them.

The nuclei of the endosperm of *Lilium* are normally pentaploid (35). Reexamination of the young seeds with twin embryos shows that double fertilization takes place. An equatorial plate stage in one endosperm was found with ± 60 chromosomes. Furthermore five nucleoli are regularly present at late telophase stages.

Gaines and Aase (81) discovered a haploid wheat plant among the offspring of a cross between *Triticum compactum humboldtii* ♀ and *Aegilops cylindrica* ♂. Normal seeds of the female parent weigh 25 to 35 milligrams, and hybrid seeds formed after the above mating are usually shrivelled and only about one-half this size. The seed containing the haploid embryo was abnormally large, weighing 45 milligrams. It is suggested by Gaines and Aase that possibly both male gametes fused with the polar nuclei, yielding the giant endosperm. Normal pistils of *Triticum monococcum* pollinated with x-rayed pollen were found (125) to yield 17.6% haploids. The finder conjectured that the treated male nuclei stimulated the egg to develop parthenogenetically and that the endosperm arose similarly or by normal triple fusion. Further study of haploids arising after the use of x-rayed *T. monococcum* pollen led to the view that, since the mature endosperms appear normally developed, the polar nuclei are fertilized (131).

Katayama (124) examined florets of *T. monococcum* collected nine days after emasculation and found small haploid embryos in a number of instances. The central nucleus had failed to divide and no endosperm was present. Later Kihara (129) worked with a strain of *T. monococcum* var. *vulgare* which gave rise to 0.5% haploid plants under natural conditions. Only diploid plants were observed after artificial pollination prior to six days after emasculation. When pollination was delayed until six to nine days after emasculation, he obtained as high as 37.5% haploid individuals. No seeds were produced from florets emasculated and not pollinated. Evidently seed formation was contingent on fertilization of the central nucleus which remained capable of fusing with a sperm

even after the embryo had begun development. These findings suggest that the increased frequency of haploids subsequent to use of x-rayed pollen (125, 131) may be due to a slowing down of pollen tube growth to such an extent that many haploid eggs divide before the sperm reaches the central nucleus.

Redinger (206) obtained a few maternal-type plants, which were assumed to be of parthenogenetic origin, from seeds borne by *Petunia nyctaginiflora* after pollination by *Salpiglossis* or *Nicotiana*. Histological study showed that, except for one tetraploid, the embryos formed after the treatment were diploid. The triploid number of chromosomes in the endosperm was established in the single case in which a count was possible. A satisfactory explanation for the origin of such an endosperm in association with a parthenogenetic embryo is not at hand. The great care which was taken in castration of the *Petunia* flowers is considered by Redinger to exclude the possibility that the seeds resulted from accidental selfing. The contention, however, that application of foreign pollen to the *Petunia* stigma stimulated such seeds to form remains open to doubt.

CULTIVATION OF EXCISED EMBRYOS IN VITRO

Broadly speaking, two periods in development of the embryo within the seed may be recognized with reference to the arrangements for nutrition. During the first of these the endosperm, in an actively meristematic condition, serves as an auxiliary to the more slowly growing and highly dependent embryo. The mother plant is host to its offspring indirectly at this time, through the medium of this special tissue. In the second period the embryo is more nearly autonomous, the endosperm either having been largely eliminated by absorption or having become a relatively passive storage tissue. The anatomical evidence indicates that the transition between these periods is gradual. The initial physiological relationship between embryo and mother plant, through the endosperm, is unique. Subsequently the embryo is much like a bud in that its sustenance is drawn directly from the maternal sporophyte, although there is no direct vascular connection. One may suppose that as the embryo enters the latter stage it has become capable of performing certain nutritive functions effectuated earlier on its behalf by the endosperm.

Excision of embryos from seeds at different stages of development and cultivation of them on artificial media offer a means of studying the functional properties of the endosperm. The embryo culture work which has been done thus far, however, is largely of a general character and has not been directed specifically toward this objective. Most of the investigations, in fact, have dealt with relatively advanced embryos which were probably past the stage of high dependence on the endosperm when removed from the seed. Experience has shown that younger embryos are much more difficult to grow in culture. No success has been reported in propagating embryos taken at the earliest stages of development. Hence little evidence is available concerning the growth requirements of embryos removed from the seed while still nurslings of the endosperm. Discovery of the special nutritive properties of the endosperm awaits the development of techniques by which juvenile embryos may be readily grown *in vitro*. It is impracticable at present, therefore, to relate the embryo culture data reviewed below directly to endosperm function. Nevertheless it is apparent that this mode of attack is capable of extension along lines which are important for a fuller understanding of the rôle of the endosperm in seed development.

Cultivation of excised embryos on nutrient media falls within the general field of plant tissue culture. White (276) has recently prepared, in handbook form, an account of the history of this comparatively new branch of physiology, its problems, experimental procedures and possibilities for further development. White points out that the only tissues which have been grown successfully *in vitro* so far possess a fundamentally meristematic character and do not have to undergo extensive de-differentiation. Apical meristems, including root tips, and intercalary meristems, such as cambium, are of this class. Fertilized eggs and embryos are meristems *par excellence*, and hence should be well adapted to artificial cultivation. Attempts to grow eggs in artificial media have failed thus far. Considerable progress has been made, however, in developing methods by which seedlings may be obtained from partially developed embryos.

The first successful effort to cultivate embryos excised from unripe seeds appears to be that of Hannig (97), working with *Raphanus* and *Cochlearia*. Hannig tested a variety of nutrient media containing sugars, mineral salts, plant decoctions, certain amino

acids, gelatin, and protein degradation products. Plants were reared from embryos only 1.2 mm. in length at the time of excision. Presumably the radicle, cotyledons and apical growing point already would have been formed in embryos of this size. Dieterich (64) found that Knop's mineral solution, to which 2.5% to 5% cane sugar and 1.5% agar were added, promoted prompt and regular growth of embryos removed from the mature, but not yet dry, seeds of a variety of plants. Embryos from certain other species, removed from the seed at the same stage and placed upon this medium, formed seedlings only after certain transformations, including disappearance of the chlorophyll, were completed. It was assumed that these latter embryos required a period of after-ripening before germination could occur. Dieterich noted that partly grown embryos excised from immature seeds tended to form seedlings directly when cultivated artificially. That is to say, the embryos skipped the stages of development which had not been completed at the time of excision. Placing the embryos below the surface of an agar culture medium was believed to favor increased embryo development and delay seedling formation. Dieterich's efforts to cultivate embryos less than one-third mature size at the time of excision were unsuccessful.

Others (71) have given particular attention to means by which asepsis of embryo cultures could be maintained. Avoidance of contamination of cultures by microorganisms is one of the principal problems with which the investigator in this field has to deal.

Cultivation of embryos on artificial media was used as a means of obtaining hybrids between *Solanum nigrum* and *S. luteum* (115). The occasional fruits which develop to maturity after this cross contain only a few very small seeds which shrivel greatly on drying. Jørgensen opened the seed coats, placed the seeds in Petri dishes on filter paper moistened with Knop's solution. A few seedlings appeared in 10 to 14 days from which adult plants were eventually grown.

Laibach (135, 136) demonstrated that the embryos within the shrunken and non-germinable seeds formed on crossing *Linum perenne* and *L. austriacum* were potentially capable of developing into vigorous plants. The mating *L. perenne* ♀ × *L. austriacum* ♂ yields fruits of approximately normal size, but the contained seeds are small and incapable of sprouting. Laibach found, however,

that if the embryos were dissected out and merely placed upon damp blotting paper, germination occurred a few days later. The fruits formed by the reciprocal cross fall before maturity. The seeds at this time do not exceed one-thirteenth mature size and are incapable of germinating directly. Both the seeds and the embryos excised from them developed further, however, if placed on cotton wadding containing 10% to 15% cane sugar. The embryos became firm, like normal embryos, in two weeks under this treatment, and when removed from the sugar solution and placed on moist blotting paper, germinated within a few days. Vigorous plants which flowered abundantly and set fruit were reared from the artificially cultivated embryos. Laibach called attention to the possibility that offspring might be obtained from various other wide crosses which gave non-germinable seeds if the embryos were excised before death occurred and were cultivated on a nutrient medium.

Laibach (137) found that the combination *Ladanum pyrenaica* ♀ × *L. ochroleuca* ♂ usually gives empty seeds. One small embryo which was obtained, however, did not respond to treatment with sugar but germinated after four weeks on Knop's solution. The seedling was weak at the start and gave a plant which was weaker than the parents. This individual, however, yielded some 100 normal appearing seeds on self-pollination.

The mating *Gossypium Davidsonii* × *G. sturtii* results in poorly developed seeds which will not sprout (227). Fourteen plants were obtained, however, by removing the seed coats from the shrunken mature seeds and growing the embryos on 5% dextrose-agar. Beasley (9) was similarly successful in rearing the hybrids *Gossypium hirsutum* × *G. herbaceum*, *G. hirsutum* × *G. arboreum* and *G. barbadense* × *G. herbaceum* on a culture medium containing 2.0% sucrose, 0.6% agar and White's (274) mineral mixture. The embryos excised from the mature shrunken seeds were carried on the nutrient medium until roots appeared and were then transferred to sterilized soil.

An embryo culture technic was used (226) to obtain offspring from a series of interspecific matings in the genus *Prunus* which had previously been found to give non-germinable seeds. The hybrid embryos were excised from the seeds when the fleshy pericarps in normal fruits of the same age were beginning their second

period of rapid enlargement and were still hard and green. The stony pericarp had begun to harden but was not yet brittle. The cotyledons were white and opaque and developing rapidly. Skirm observed that embryos which had begun to break down in the seed did not recover in the culture medium. The excised embryos were placed for half their length in a 1% agar medium containing Knop's mineral mixture and 1% to 3% sucrose or glucose. A small amount of a trace solution, the composition of which is not given in the author's report, was added also. Using similar methods, Skirm obtained seedlings of several *Lilium* interspecific hybrids which had proved non-viable under natural conditions. Among these was the hybrid *L. Henryi* \times *L. regale* which had not been reared previously in spite of numerous attempts to obtain it by conventional means.

Seeds obtained from the cross *Hordeum jubatum* \times *Secale cereale* cease growth within 12 days after fertilization, and the embryo in the mature caryopsis is inviable. This intergeneric hybrid has recently been reared by excising the embryo from the seed just previous to collapse and cultivating it on a 0.5% agar medium containing 2% sucrose, White's mixture of mineral salts and a small amount of yeast extract (22). Eighty-one embryos 9 to 12 days old were placed in culture. Forty-seven were lost as a result of contamination. Of the remainder, 33 made a callus-like growth on the nutrient medium. Only one embryo formed a seedling which was potted and grown to maturity.

Others (50, 153) have reported briefly upon the use of artificial embryo cultures in obtaining new interspecific hybrids in *Datura*. Eleven of 14 crosses of *D. ceratocaula* with other herbaceous *Datura* species yielded dissectable embryos. Eight hybrids have grown in culture. Three of these have been successfully carried forward to soil and two have been grown to maturity. *D. innoxia* \times *D. discolor* also has been reared to maturity after cultivation at the embryo stage in an artificial medium.

Seeds of early ripening varieties of sweet cherry (*Prunus avium*), peach (*P. persica*) and plum (*P. domestica*) cease growth before full development is attained. Rarely are such seeds capable of germination under standard horticultural conditions. Tukey (255) and Tukey and Lee (258) have carried out a comprehensive series of investigations on the physiology of seed and fruit formation in

these important classes of orchard plants during the course of which they have developed efficient methods for rearing the offspring of early varieties. Tukey (256) removed the embryos from the seeds on attainment of the maximum size characteristic of the variety, cultivated them on nutrient media, and transplanted the seedlings to pots in the greenhouse when true leaves appeared. The stony pericarp was split open and the embryo freed from the integuments, nucellus and endosperm. After sterilization for five minutes in a 2% solution of chlorine (277) the embryos were placed on a nutrient agar medium in screw-top vials just large enough to accommodate the resulting seedling. Various salt solutions whose concentrations varied over a range of one- to 10-fold were successfully used. Two per cent of glucose was optimal for young embryos and lower concentrations for older embryos.

Excised *Prunus* embryos in culture do not pass through the stages which characterize the further development of normal embryos on the mother plant. Tukey (257) observed that instead they entered at once upon an independent development varying in character with age at the time of excision. Peach embryos, excised prior to 51 days after full bloom, failed to develop further in the above culture medium. Vigorous roots developed from embryos excised at 87 days, but the central axis of the epicotyl remained relatively short and was terminated by a rosette of green stipule-like appendages. Shoot development from 94-, 105- and 108-day embryos was abnormal also. Dwarfish seedlings with vigorous roots and broad leaves were obtained from 118-day embryos. The shoots of these latter plants began normal elongation after being held for 30 days in subdued light at 45° F.

Blake (11) used a somewhat different procedure for germinating the shrivelled seeds of early ripening peach varieties which do not readily respond to ordinary stratification methods. The kernels were removed from the stones as soon as the fruits ripened and placed in pint Mason jars containing quartz sand, sugar and a mineral salt solution. The jars then were held in the dark at about 40° F. for 12 weeks, during which time roots formed and the cotyledons began to open. Following removal of the cultures to a greenhouse, seedlings 1½ in. to 2 in. high developed in about three weeks. The seedlings were then potted and prepared for transplanting to the field. By these methods Blake secured fruiting

trees from 75% of the seedlings in three years after the seed was taken.

Using cultural methods patterned after those of Tukey, Lammerts (138) developed a schedule which enabled him to bring hybrid peach trees to flower in only two years after the cross was made. Addition of vitamin B₁ at the rate of 1 mg. per liter to a 0.9% nutrient agar medium was found helpful in stimulating root formation. One-half per cent sucrose for mature and 2.0% sucrose for immature embryos was used. The culture bottles were placed in subdued light from the start. The embryos began growth soon after placing them in culture and were usually large enough to transplant in two or three weeks. Many seeds from crosses between certain varieties, not characterized by any particular season of maturity, did not respond to the embryo culture methods used. Some of these embryos grew after exposure in culture bottles to a temperature of 40° F. for six weeks.

Delayed germination of seed is a major problem in the propagation of many species of plants. The delay in certain cases is due to dormancy of the embryo, apparently associated with growth-inhibiting substances formed in some part of the seed. Separation of the embryo from the tissues normally enclosing it, followed by cultivation of the embryo on an artificial medium, has proved to be an effective means of surmounting this particular block to continued development.

Tukey (256), working with early varieties of the sweet cherry, found that embryos enclosed in the endosperm, nucellus and integuments did not grow in culture unless the swelling of the embryo was sufficient to burst the integuments and expose the cotyledons to light. Normally developed *Iris* seed, when sown in the soil, germinates over a period of three years (272). By artificial cultivation of excised embryos, seedlings were obtained in a few days. A procedure was also worked out whereby about 95% of the embryos excised from mature *Iris* seeds, and grown in a nutrient medium, yielded seedlings (204). Granular sodium hexametaphosphate (Calgon) was used as a source of phosphorus in a 0.7% agar medium containing 2.0% cane sugar, together with other mineral salts. The hexametaphosphate forms a soluble complex with both iron and calcium. Embryos completely detached from the endosperm germinated promptly in the medium. Growth was inhibited,

however, if even a small portion of endosperm tissue remained in contact with the embryo. Randolph (203) has recently described an improved method of cultivating embryos excised from *Iris* seeds showing delayed germination. McLean (153) notes that *Datura ceratocaula* seeds ordinarily give about 0.1% germination. Practically complete germination was secured, however, by cultivation of the excised mature embryos in a nutrient medium.

Cultivation *in vitro* of proembryos and very young embryos presents special difficulties. Few investigators have succeeded in obtaining seedlings from these juvenile structures. Either the minute embryo dies in culture or growth results in the formation of callus-like tissue only. Some progress has been made, however, in discovering the conditions required for development at these early stages.

Evidence has been brought forward (259-262) for the existence in raw coconut milk of a thermolabile substance which in very low concentrations promotes the growth of young embryos. This substance has been provisionally termed "embryo factor". Embryos as small as 0.2 mm. in length and as young as 10 days excised from *Datura stramonium* seeds were reared on a medium containing 0.7% agar, 2% sucrose, a mixture of nutrilites (conc. in mg. per liter: glycine, 3.0; thiamin, 0.15; ascorbic acid, 20.0; nicotinic acid, 1.0; vitamin B₆, 0.2; adenine, 0.2; succinic acid, 25.0; pantothenic acid, 0.5), 0.01 M phosphate buffer, and three drops of raw coconut milk per 0.75 cc. *Datura* embryos smaller than 0.5 mm. in length when excised from the seed usually do not develop on the above medium, according to van Overbeek, unless coconut milk is included. Increases of 500 times the initial volume of tissue in six days were observed on the complete medium. It is significant that embryos cultivated on coconut milk medium grew and differentiated as embryos for at least 10 days, root formation remaining suppressed, as in the seed. Embryos pre-cultured in the complete medium formed roots on being transferred to a similar medium free of coconut milk. Young embryos were found to require a higher concentration of coconut milk in order to continue growth than embryos excised at later stages. Evidence was obtained for the presence in coconut milk also of a heat-stable factor promoting callus-like growth and no differentiation and of a heat stable factor which inhibits root growth and may be related to auxin.

Others (13) report that powdered malt extract is an effective substitute for the embryo factor from coconut milk if sterilized by filtration instead of by heat.

A purified extract of coconut milk was obtained (263) which had an activity 170 times that of raw coconut milk. This preparation aided the growth of *Datura* embryos on artificial media at a dilution of 1:19,000. Extracts of *Datura* ovules, wheat germ, almond meal and yeast showed embryo factor activity also. The investigators found that the optimal pH of the nutrient medium for *Datura* embryos was 7.0 during the first two to four days of growth and 5.5 during later development. A temperature of 32° C. was more favorable than 25° C. and 36° C.

A method of cultivating embryos of maize excised at eight to 10 days after pollination and 0.3 to 3 mm. in length has also been described (91). Embryos between 0.3 and 1 mm. in length require a sucrose concentration of 5% and fail to grow in more dilute media. Asparagine added at the rate of 1.5 grams per liter of culture medium accelerates embryo growth, particularly of the epicotyl. Maize embryos excised at 10 days and longer than 0.3 mm. were found not to require coconut milk for continued growth. Smaller embryos did not survive in the medium used even with the addition of coconut milk.

White (275) suggests that oxygen gradients may be an important factor in controlling differentiation. He found that cultures of callus from a *Nicotiana glauca* × *N. langsdorfii* hybrid which had been maintained in an undifferentiated state for 20 weeks or more on a semi-solid 0.5% agar medium formed leafy branches following immersion in a liquid nutrient.

La Rue (142, 143) succeeded in growing complete plants not only from immature embryos but also from various part of such embryos. The basis of the medium used was White's (274) solution of mineral salts to which 1% agar and 2% cane sugar were added. It was found necessary to supplement this mixture with either heteroauxin or an extract of dried brewer's yeast in order to obtain growth. The culture media, tubes and dishes were autoclaved, but the tissues were given no sterilization of any kind for fear of injury. Contamination proved to be a troublesome factor.

Species differed in the ease with which their excised embryos could be grown on this medium. Embryos of *Taraxacum officinale*,

Chrysanthemum leucanthemum, *Lactuca canadensis*, *Coreopsis lanceolata*, *Lycopersicon esculentum*, *Nicotiana tabacum* and *Bryophyllum crenatum*, as small as 0.5 mm. to 1.0 mm. in length, were grown to the seedling stage. Unsuccessful attempts were made to cultivate embryos excised from immature seeds of *Alyssum*, *Plantago*, *Malva*, *Eschscholtzia*, *Lathyrus*, *Epilobium*, *Asclepias* and *Melilotus*. Maize embryos slightly longer than 0.5 mm. and *Avena* embryos about 1.0 mm. in length were grown into plants capable of living in soil. The size of the maize seedlings obtained varied with the size of the embryos at the time of excision. Monocotyledonous embryos, whatever their stage of development when excised, ceased embryonic development when placed on agar media and grew directly into seedlings.

La Rue's (143) success in rearing plants from pieces of embryonic tissue are of much interest. Dandelion, wild lettuce, ox-eye daisy and tomato were used. Complete plants were reared from cotyledons excised from immature embryos of tomato, although in the other species cotyledons failed to regenerate. Roots formed on segments of the upper ends of hypocotyls, and basal segments of hypocotyls with radicles intact grew new buds on the cut ends. Midsections of hypocotyl, lacking both plumular and radicle meristems, produced new buds on the upper ends and new roots on the basal ends. Polarity of the excised segments of embryo did not change.

Embryos of *Zizania aquatica* excised when 0.2 to 1.5 mm. long from seeds 5 to 11 days old were successfully grown on a medium similar to the above but lacking yeast extract (144). Small embryos required a longer time than larger ones to reach the seedling stage. Embryos smaller than 0.4 mm. grew atypically and failed to form seedlings. The investigators observe that growth in culture is characterized by precocious formation of the shoot and retarded growth of the primary root. This difference is less in evidence or even absent with older embryos.

Merry (167) observed that the cells of immature barley embryos cultivated on an agar medium containing 2.0% sucrose and Shive's mineral solution undergo considerable enlargement. The cells in normally growing embryos tend to remain small. Furthermore, mitosis occurs with greater or lesser frequency in all parts of the embryo within the seed. The capacity for cell division appears to

be lost, however, in some parts of the cultivated embryos and growth is restricted to the regions in which mitosis occurs.

The difficulties which have been encountered in the cultivation on artificial media of embryos excised from young seeds suggest that the endosperm of the latter performs some special nutritive function which is not readily duplicated on artificial media. Possibly the capacity of the endosperm to nourish juvenile, in contrast to older, embryos is transitory, characterizing the tissue while in a meristematic condition but disappearing as the structure matures and storage occurs. A fuller knowledge of the growth requirements of young embryos may be expected to contribute significantly to an understanding of endosperm physiology.

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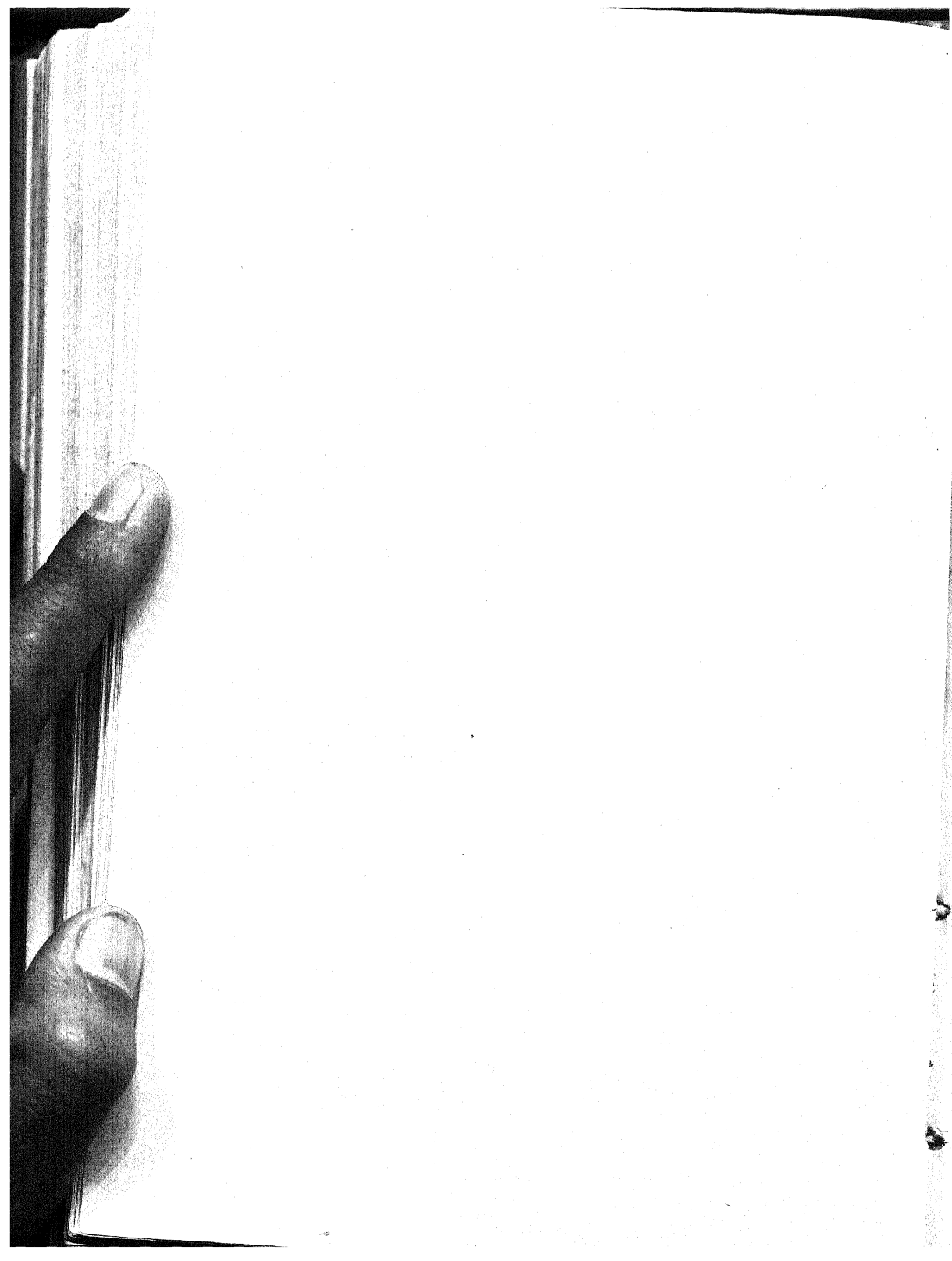
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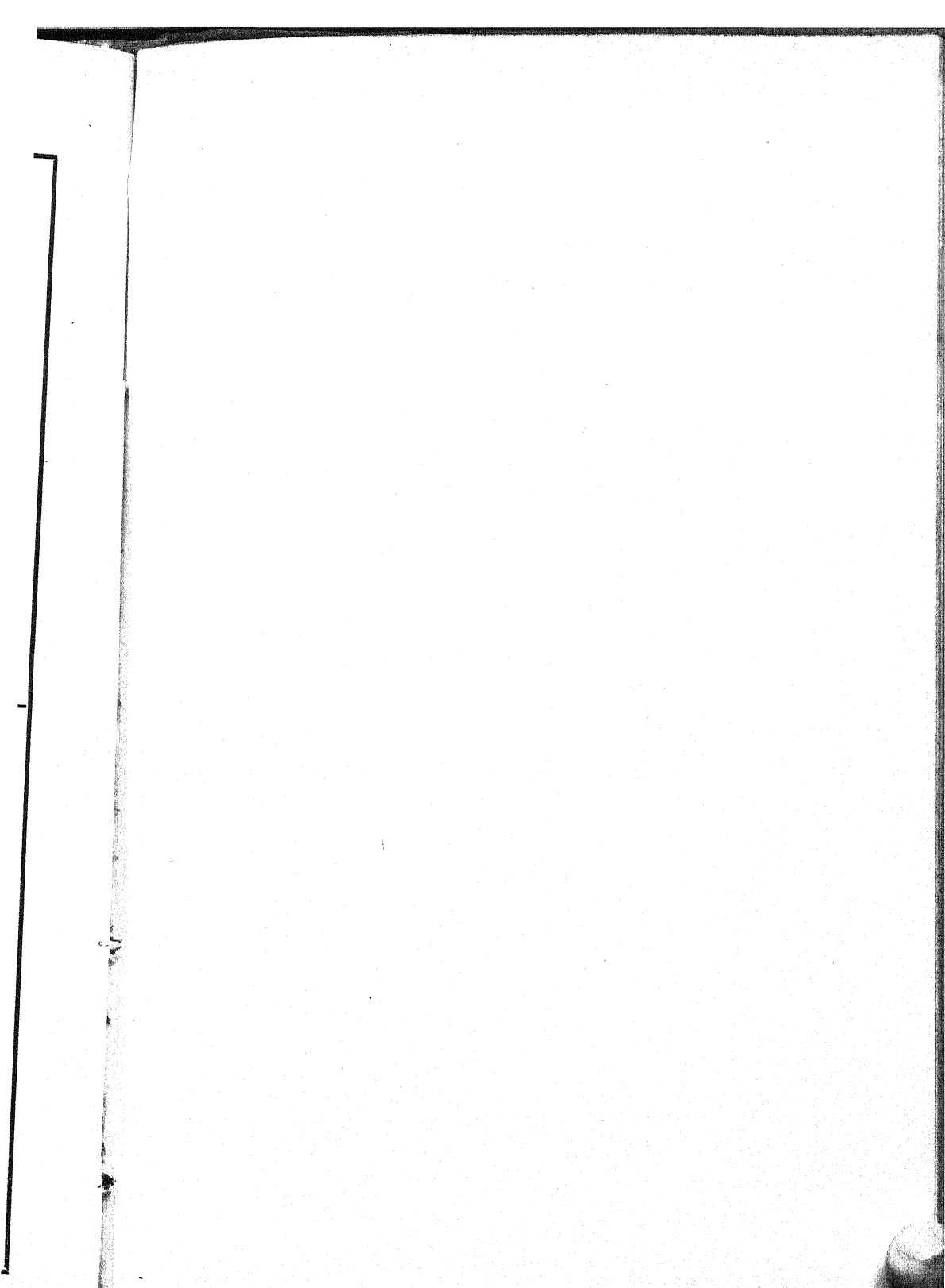
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THE PHYSIOLOGY AND BIOCHEMISTRY OF RUBBER FORMATION IN PLANTS

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INTRODUCTION

It is the purpose of this review to survey the present knowledge concerning the production of rubber by plants, especially the factors, environmental and internal, which influence rubber production, and the particular chemical reactions which lead to formation of the rubber hydrocarbon. There are today three rubber-forming plants concerning which a considerable amount is known: *Hevea brasiliensis* (Muell.), *Taraxacum kok saghyz* (Rodin) and its allies, and *Parthenium argentatum* (Gray), the guayule. Smaller bodies of data have been collected regarding certain other species, especially *Solidago* sp. and *Cryptostegia grandiflora* (R. Br.), while still less is known about the physiology of the great majority of rubber-forming plants. In this review attention will be mainly centered on the five species named above, although data obtained from other rubber-producing plants will be introduced when pertinent and available. Since this review is to concern primarily the physiology of rubber formation, no attempt has been made to cite every paper dealing with rubber-bearing plants. A bibliography of the literature on rubber-bearing plants other than *Hevea* has already been prepared (155).

Survey of the Principal Species

Formation of rubber appears to be a quantitative rather than a qualitative character of plants. Thus we find species which contain no rubber, those in which rubber is a major constituent (22% or more on a dry weight basis in guayule (140)), and all possible intermediates. In the vast majority of rubber-forming species the amount of rubber in the plant or obtainable from it is small. Of the 1,791 species reported to contain rubber (155), only 554 have

been used as rubber producers. Wiesner (225) lists 490 species as being of greater or less importance as rubber-bearing plants. Of these only a few produce rubber in sufficiently large quantities and in sufficiently pure form to be considered as even potential commercial producers. A list of the more important is given in Table 1, included in which are also a few rubber-forming species which, while unimportant at the present time, are of interest in that they occur in temperate regions.

Rubber formation is a property scattered through numerous families of the plant kingdom in no discernibly regular fashion. The Moraceae, Euphorbiaceae, Apocynaceae, Asclepiadaceae and Compositae are particularly well represented. All genera within any one family are not ordinarily rubber-forming, and the species of one genus may differ greatly in rubber-forming capacity, as, for example, in *Ficus* and *Euphorbia*. On the other hand, certain rules regarding distribution of rubber have emerged. Rubber formation, for instance, is a property confined to the dicotyledonous branch of the angiosperms and is not found in monocotyledonous plants, in gymnosperms or in lower plants. The great majority of rubber-forming species are tropical in distribution. Within a single genus, as *Euphorbia*, the tropical representatives may include numerous species which form and accumulate considerable amounts of rubber, whereas the representative of this same genus in the temperate zone may form, in general, little or no rubber. Temperate zone species which form sufficient rubber to be considered as potential rubber sources are few, namely, *Parthenium argentatum*, *Taraxacum kok saghyz* and its allies, and perhaps *Asclepias erosa* (Torr.) as well as other species of this genus. The possibility that new and important rubber plants will be found in the future, while remote, is not to be excluded. Although the great majority of the more common species have been investigated, it should not be forgotten that the discovery of *Taraxacum kok saghyz* in the remote Tien Shan mountains of Russian Central Asia occurred as recently as 1931.

The rubber-like hydrocarbon, gutta percha, is produced by a small number of species, among which are *Palaquium Gutta* (Burck), source of the gutta of commerce; *Mimusops balata* (Gaertn.); *Achras sapota* (L.), source of chicle, an impure gutta; *Dyera costulata*, source of jelutong; *Cnidioscolus elastica* and *C. tepiquensis* (125), sources of chilte, both substitutes for chicle; *Euonymus*

TABLE 1
SOME SPECIES OF RUBBER-FORMING PLANTS
The number of temperate zone species here listed is unduly large in proportion to the tropical species.

No.	Species	Family	Native habitat	Habit	Reference*
1	<i>Asclepias</i> sps.	Asclepiadaceae	No. America	Herb	17, 23, 24
2	<i>Castilleja</i> sp., or <i>elastica</i> , Ulci	Moraceae	Cent. America	Tree	27, 144
3	<i>Chrysothamnus nauseosus</i> (Britton)	Compositae	No. America	Shrub	61
4	<i>Cryptostegia grandiflora</i> (R.Br.)	Asclepiadaceae	Trop. Africa	Vine	171
5	<i>Euphorbia Intisy</i> (Drake)	Euphorbiaceae	Madagascar	Shrub	201
6	<i>Ficus</i> sp., or <i>elastica</i>	Moraceae	Asia; Africa	Tree	144
7	<i>Funtumia elastica</i> (Stapf.)	Apocynaceae	West Africa	Tree	144
8	<i>Hancornia speciosa</i> (Gomez)	Apocynaceae	So. America	Tree	144
9	<i>Hevea brasiliensis</i> (Muell.)	Euphorbiaceae	So. America	Tree	58, 144
10	<i>Landolphia</i> sp.	Apocynaceae	Africa	Vine	144
11	<i>Mamhot Glaziovii</i> (Muell.)	Euphorbiaceae	So. America	Tree	144
12	<i>Parthenium argentatum</i> (Gray)	Compositae	Mexico; Texas	Shrub	141
13	<i>Sapium</i> sp.	Euphorbiaceae	So. America	Tree	144
14	<i>Scorzonera tau saghyz</i> (Lipsch. et Bosse)	Compositae	Cent. Asia	Herb	105, 136
15	<i>Solidago</i> sp.	Compositae	No. America	Herb	169
16	<i>Toraxacum kok saghyz</i> (Rodin)	Compositae	Cent. Asia	Herb	51, 110

* The references listed are conveniently available rather than original sources.

species and *Eucommia ulmoides* (Oliver). The last two are temperate-zone species which have been used for commercial production of gutta in the Soviet Union.

Latex

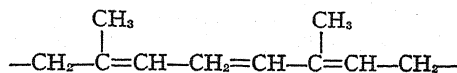
Rubber frequently, but not always, occurs in the plant in the form of minute particles suspended in a liquid matrix, the whole forming a latex. The latex is in turn contained within more or less specialized lactiferous cells or vessels. The milky appearance of a latex does not depend directly on the chemical composition of the suspended matter, but is due merely to the fact that the matrix contains many small particles with an index of refraction greatly different from that of the dispersion medium. A latex need not contain rubber, but may contain suspended matter of various kinds, as, for example, proteins in *Ficus callosa* (210) and waxes in *Brosimum Galactodendron* (D.Don) (75). At the beginning of the rubber emergency various temperate-zone species of *Euphorbia*, *Poinsettia*, *Lactuca*, *Asclepias*, etc. were widely suggested as possible rubber sources, principally on the basis of latex content. With few exceptions, the latices of these species contain negligible amounts of rubber; more usually they contain considerable quantities of tri-terpenes or their derivatives.

Latex particles vary from 0.01 to 50 or more microns in diameter in various species. Within any one latex, particles of various sizes occur, and, in addition, the particles may vary from spherical to pear- or rod-shaped. It has been shown with the aid of the electron microscope that the larger particles are made up by the union of smaller ones (90). In *Manihot* rod-shaped particles are achieved by polarized union of the spherical ones. A vast variety of compounds occurs in the dispersion medium of various latices, e.g., starches, sugars, amino acids, sterols, alkaloids, organic acids, etc. The general physiology of latex has been reviewed (154), and further information may be found elsewhere (75, 88).

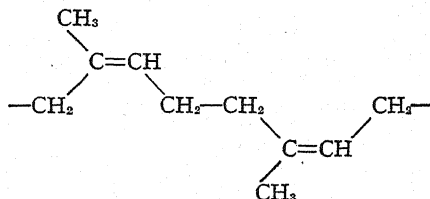
Structure of Rubber and Gutta Percha

Rubber and the related gutta percha consists of long chains of isoprene residues linked together through 1:4 linkages (Fig. 1a). The number of isoprene residues per molecule in rubber may be 1,000-3,000 or more, while in gutta it is probably somewhat less

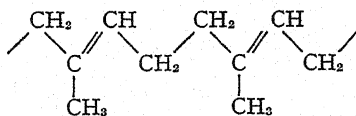
(195, 196). In the unstretched state rubber is amorphous, but it may be caused to crystallize by a protracted stay at 0° C. or by stretching. During stretching the elongated rubber molecules assume positions in regular pattern, parallel to the direction of stretch. It has been shown (103) that stretched rubber yields X-ray interference diagrams, and following this observation the crystal structure of rubber has been worked out (52, 146). The identity period along the molecule is 8.1 Å and contains two isoprene units. The double bonds are all of the *cis* configuration (Fig. 1*b*). Gutta percha differs from rubber in that it is plastic rather than elastic at



1a. Generalized form of polyisoprene chains in rubber and gutta percha.



1b. Polyisoprene chain with double bonds in *cis* position as in rubber.



1c. Polyisoprene chain with double bonds in *trans* position as in gutta percha.

FIG. 1. The polyisoprene chain and its two isomers as they occur in rubber and in gutta percha.

ordinary temperatures. The native or α -gutta percha when heated to the melting point (about 60°) and cooled rapidly assumes a new form, β -gutta. Both forms are microcrystalline at ordinary temperatures. In β -gutta the identity period along the molecule is 4.8 Å and consists of one isoprene unit in a somewhat more elongated state than those of rubber. The double bonds of gutta are all in the *trans* position (Fig. 1*c*). The structure of amorphous unstretched-rubber has been suggested (129) to be one of randomly curved and curled molecules. Bunn (53), on the other hand, has adduced evidence that the rubber molecules are arranged in bundles or crystallites. Since the individual molecule is much longer than

the crystal, any one molecule must pass through several crystals, a concept similar to that developed for cellulose (42, 76). In amorphous rubber Bunn (53) suggests that the lattice within the crystal is disordered by thermal movements or wriggling of the molecules. More complete orientation within the lattice can be attained either by lowering the temperature or by stretching of the system. Bunn attributes the crystalline state of gutta at ordinary temperatures to more restricted thermal motions (wriggling) of the gutta molecules, owing to steric factors associated with the trans configuration. The thermal motions of the isoprene chain appear also to be of great importance in relation to the elasticity of the material. At temperatures above its melting point, where thermal motion is greatly increased, gutta becomes elastic, although still to a less extent than rubber. Although the trans isomer, gutta, is thermodynamically more stable than rubber, no evidence of the conversion of rubber to gutta in the plant could be found (91). All species examined contained either rubber alone or gutta alone.

HEVEA

Introduction

Of the several species of the genus *Hevea* (Euphorbiaceae) not more than two or possibly three can be regarded as effective producers of rubber, and only one, *H. brasiliensis*, has become of commercial importance. This species is found in the native state in the valleys of the Amazon and its tributaries. Rubber was obtained from the native trees by the South American Indians in pre-Columbian times, and later (1850-1910) a major portion of the rubber of commerce was obtained from wild plants. Seeds of high-yielding trees collected in Brazil by Sir Henry Wickham in 1876 were germinated at Kew, England, and 2,000 seedlings sent to Ceylon, whence *Hevea* was distributed throughout the Old World tropics. The first commercial production from the new plantations occurred in 1910, and within three years this production rivaled that produced from all the wild trees of South America. In recent years *Hevea* has been grown principally in Sumatra, Java and Malaya. The South American leaf blight, which is coextensive with the natural distribution of *Hevea*, has limited production in the New World, although selection of disease-resistant high-rubber-yielding strains holds promise for the future production of *Hevea* rubber in Latin America (50, 181).

Although an immense amount of work has been done on the production of rubber by *Hevea brasiliensis*, only a minor portion of the effort has been directed toward a study of the physiology of rubber formation. Most workers, on the contrary, have concentrated on the agronomic or technological aspects of commercial rubber production or upon the phenomena of latex flow. As a result, the published data available on the physiology of rubber production in *Hevea* are actually less than those available for other less important rubber-producing plants, such as kok saghyz and guayule.

Amount of Rubber Produced

Statistics (212) based on 1,600,000 unselected trees of all ages show a modal production of about 1.5 kg. of rubber per tree per year in East Indian plantations. Among these trees considerable numbers of individuals yielded as little as 0.7 or as much as 3.4 kg. per tree per year. At the age of 16 years, plantings of unimproved stock yielded an average of 380 lbs. of rubber per acre per year. Productions of over 1,000 lbs. of rubber per acre per year are obtained from plantings of trees budded from high-yielding stock, and productions of over twice this are said to be expected (212). Although tapping may be started with trees as young as four years, the yearly production increases rapidly to reach a maximum at an age of 15 to 20 years.

Anatomy and Rubber Production

The anatomy of *Hevea* has been reviewed (36, 212). The rubber-bearing latex is contained within latex vessels, of which the most important are those produced by the cambium and which are hence of secondary origin. These vessels, produced in successive rings in the bark, extend the length of the trunk but are also extensively anastomosed in the tangential direction. Only a small number of connections are found between the vessels of different rings. The vessels of any one ring form, then, a continuous anastomosed system or mantle from which latex may be drained by tapping. The vessels actually ascend the trunk at an angle slightly inclined to the vertical, *ca.* 4°. The bark is traversed in the radial direction by lens-shaped rays of parenchymatous cells. In the older portion of the bark, scattered groups of these cells undergo radical cell-wall thickening and become groups of stone cells, a process which results in breaking the continuity of the latex vessels. The vessels of the

older portion of the bark are for this reason unproductive, so far as latex yield is concerned. The parenchyma also serves as a reservoir of reserve carbohydrate, especially starch, at the expense of which rubber is formed. Phloem is found particularly in the neighborhood of the latex vessels. Phloem of the current growth is in direct connection with that of the current leaves, while the older phloem is not.

A vast number of experiments on tapping methods have been carried out with *Hevea* (128, 211). It appears desirable to tap a tree not more than half the days of the year, which may be accomplished by tapping every other day or by tapping every day for a period followed by an equal period of rest. Tapping is commenced at about one meter, more or less, above the ground level. A cut ascending to the left is made over one-third to one-half of the plant's circumference. This groove is cut to within one-half mm. of the cambium, and is renewed at each tapping by removal of approximately one mm. of bark from the lower edge. Thus during tapping the tapping surface gradually moves downward. After one year a fresh groove is cut and a different panel tapped. The established superiority of basal tapping to tapping high on the tree may be related to the fact that the maximum number of rings of latex vessels occurs at the ground level, the number decreasing with increasing distance up the tree. In budded seedlings, anatomical junction of the latex vessels of scion and stock are affected, since latex can move from scion to stock (213). The vessels, which are formed by dissolution of end walls of contiguous cells, are living and polynucleate. There appears to be no sharp demarcation between cytoplasm and cell sap, although cytoplasmic material does appear to be more concentrated at the periphery of the cell (38).

Formation of Latex Particles

That the latex particles are probably formed *in situ* in the bark, rather than transported from the leaves, has been shown by Fitting (70) and by Bobiloff (32-35). The latter showed that formation of rubber may take place in an isolated strip of bark as well as below the ring in girdled trees and in stumps. That latex is not transported from the leaves is also shown by the fact that there is no connection between the latex vessel systems of leaves and bark (212). It has also been revealed that in trees whose tops represent

a strain different from the trunk, the latex particles characteristic of the top are never found in the trunk (87). The fact that rubber is exclusively in the lactiferous elements indicates strongly that the material is synthesized in these cells. Direct microscopic observation of the formation of latex particles in the cytoplasmic layer of the latex vessels of *Ficus* and other species has been reported (38). The particles once formed gradually escape into the central vacuolar space. Similar observations have been made with the aid of vital stains (172).

✓ *Physiology of Latex Flow*

The physiology of latex flow has been treated in detail (17, 73, 74, 212). Frey-Wyssling has calculated, on the basis of anatomical measurements, that resistance to the flow of latex in the longitudinal direction is about one-ninth as great as it is in the tangential direction. This has enabled him to establish lines of flow along which latex should move toward a tapping cut. In a downward tap of the kind described above, latex should flow from below and to the sides of the incision as well as from the bark regions to the right and left and above the incision. This conclusion confirms that obtained experimentally in various ways by several investigators (17, 213). During the actual flow, latex is drained from regions one meter or more from the cut. Over a tapping period of months latex is probably drained from even larger areas of the bark (212).

During regular tapping the concentration of rubber in the latex may diminish from an initial 50% or more on the first day to 40% or less after a few days or weeks (73, 58). This decrease in concentration is attended by an increase in volume of latex produced per tapping, so that for the first few tappings the total rubber obtained per day actually increases. Not only does the latex become successively more dilute with successive tappings, but also during any one tapping the first latex is more concentrated than the later portions, a phenomenon known as the dilution reaction (17). This phenomenon has been elegantly treated by Frey-Wyssling (74, 73). The latex vessels in the intact state are under turgor (the latex normally has an osmotic pressure of about 10 atm. (212)) and are at the same time in osmotic equilibrium with the surrounding cells, the wall or turgor pressure of the latex vessels just balancing the osmotic pressure. When the tapping incision is made, the turgor of latex vessels is reduced at the point of incision, the osmotic equi-

librium is upset, and water is taken up by the latex from the surrounding tissue at the same time that the latex flow is taking place in response to the turgor gradient.

Decrease of rubber concentration and increase in latex volume after initiation of tapping has also been explained (212) on the basis of dilution reaction during the first tappings. The thick relatively viscous latex flows only slowly, and only a small area of bark is drained at the first tapping. As the latex is thinned by the dilution reaction, larger and larger areas of bark are drained. The reverse of the dilution reaction, *i.e.*, a thickening of latex, takes place after the latex flow has ceased. This has been studied quantitatively by Arisz (15), who has shown that in addition to a reestablishment of osmotic equilibrium between latex vessel and parenchyma cell, thickening is accomplished also by secretion of new rubber. In one case, for example, Arisz calculated that more than 0.3 gm. of rubber was formed per 100 cc. of latex per hour. The fact that rubber is regenerated so rapidly after tapping has led to the conclusion (75) that the rubber-forming system presumed to be contained in the cytoplasm is not drained from the vessel during latex flow but is retained intact or nearly so. Similarly in untapped trees the rubber concentration again increases to the initial high level. The factors regulating rubber concentration in the latex of untapped trees are unknown.

Constitution of Hevea Latex

The constitution of *Hevea* latex varies with the water content of the plant, the season of the year and other factors (75). For this reason the quantities or concentrations of various substances given below as being present in latex are to be regarded as approximate only. The outstanding component of *Hevea* latex is rubber, which is present to the extent of 20–60% of the fresh weight of the latex and which occurs in the form of latex particles. These particles range in size from the limits of microscopic resolution to one to three microns in diameter and from spherical to pear-shaped in form (38, 123, 124). Small spherical particles are characteristic of latex from young trees or young parts of the tree, whereas pear-shaped particles are more typical of latex from mature trees (87). Examination of *Hevea* latex with the aid of the electron microscope has revealed the presence of particles as small as 0.03 micron in diameter (90). Such particles would contain only one rubber

molecule of molecular weight, circa 1,000,000. The electron microscope pictures also reveal clearly that larger latex particles are made up by successive fusion of the smaller basic particles. Investigations of latex particles with the aid of a micromanipulator (72, 87) have suggested that the particles contain a more liquid interior and a more solid outside layer. Hauser (89) stresses the possibility that the same kind of molecules may occur in the sol and gel form in the interior and on the surface of the particle, respectively. Others (90) believe that the observed properties of the latex particle can equally well be explained on the basis of a homogeneous mass made up of long, entwined molecules of rubber. The latex particle is, lastly, covered by an adsorbed sheath of protein which contributes to its observed properties of electrophoretic mobility as well as to the coagulation properties of the latex (88). In addition to rubber particles, *Hevea* latex contains drops of resin and the nuclei of the latex vessels.

Aside from rubber, whole latex contains 0.3–0.7% of ash, 1–2% of nitrogenous compounds, approximately 2% of so-called resins and 1–2% of sugars and sugar-like compounds (58). The residue left after removal of the particulate matter from latex is known as serum. This serum contains 0.3–0.5% protein whose composition has been investigated (5, 7, 8, 206, 207). The portion of protein which is adsorbed on the rubber particles and removed with them is apparently similar in composition to the residual serum proteins (206). The serum protein yields on hydrolysis alanine, aspartic acid, dihydroxyphenylalanine, glutamic acid, histidine, leucine, ornithine, hydroxy proline, tyrosine and valine (7, 8). Electrophoretic analysis has shown that *Hevea* serum contains one principal protein as well as six other proteins in lesser amounts (183). These proteins may include enzymes, since latex serum has been shown to possess peroxidase activity (191).

Free amino acids occur in small amounts in latex serum. The following have been identified: tyrosine, leucine, isoleucine, valine, arginine, aspartic acid, proline and phenylalanine (220). Altman (6) reports, in addition, the presence of glycine, alanine, cysteine and glutamic acid. Ammonia, trigonelline, methyl amine and choline also appear to be present (9).

Altman and Kraay have isolated a lecithin from latex (11). Qualitative tests indicate that, in addition to glycerophosphoric acid

and choline, several different fatty acids were involved, *e.g.*, palmitic, stearic, arachidic, oleic and linoleic. This lecithin has been estimated to make up to 0.03% of the latex (208). Small amounts of free sterols, resin acids and wax are also present (10).

Hevea latex contains 0.5–2.0% of quebrachitol (methyl inositol) (see 58). The concentration of this compound undergoes variations correlated with the physiological status of the tree. This behavior suggests that quebrachitol may be mobilized as a reserve substance and may hence participate in the metabolism of the plant. Up to perhaps 0.35% of reducing sugars is also present in the latex (58).

A high degree of negative correlation has been found between rubber concentration in *Hevea* latex and the concentration of acetone- and benzene-insoluble material (197). Thus latex of seedlings or leaves which is low in rubber was found to be high in insolubles. The compound or compounds responsible for this behavior have not been identified. Similar results were obtained by the same authors with *Cryptostegia grandiflora* (198). Here a crystalline material, responsible for the correlation, was isolated from the acetone- and benzene-insoluble fraction.

Significance of Rubber for Hevea

No rubber utilization by *Hevea* seedlings growing in the dark after removal of the seed could be detected (39), even after all starch had disappeared from the plants, and respiration of *Hevea* was found to be independent of the presence of latex (37).

Effect of Environmental Factors on Rubber Formation by Hevea

Nutrition. The early work on the mineral nutrition of *Hevea* has been reviewed (83). Nitrogen or phosphate deficiencies are the ones most likely to be met with in plantation practice. Application of nitrogen as ammonium sulfate, in general, results in increases of growth rate of the young tree and in increases of yield of latex in mature trees. Phosphate deficiency may, however, occur, especially in young trees (84). Application of foliar analysis to the study of the N and P nutrition of *Hevea* has been described (55). A high degree of correlation was found between N content of the leaf and growth rate as well as latex yield in low N soils. Similarly on soils low in available P, significant correlations between leaf P and growth rate and latex yield were found.

Light. Direct intervention of light is not needed for rubber synthesis in *Hevea*, since rubber can be formed, as noted above, in leafless stumps. This rubber is formed presumably at the expense of stored carbohydrate. Defoliation of the entire tree does, however, result in lowered rubber production over a year's time (187).

Root Stock. High-yielding scions grafted onto high-yielding seedlings are said (212) to be better rubber producers than similar scions grafted to low-yielding root stocks. It was mentioned above that latex flow between stock and scion has been established, so that this influence of the root stock may be in part attributable to actual participation of the root stock in rubber production, even though it is not itself tapped. Whether or not other factors are involved is undetermined.

Season. *Hevea* undergoes a loss of leaves once a year, generally in the dry season (58). The trees remain bare only a short time, after which new leaves are produced. Although many trees undergo this "wintering", as it is known, at regular intervals of one year, other trees may have longer or shorter cycles (16). A minimum yield of latex generally accompanies the production of the new leaves, and a maximum flow follows in about two months (16).

RUSSIAN DANDELION

Introduction

In the late 1920's the Soviet Union, desirous of becoming economically self-sufficient in natural rubber production, began an intensive search for native rubber-bearing plants. From among the several thousands of plants investigated, kok saghyz, the Russian dandelion, has emerged predominant. In this review kok saghyz will be considered together with its related rubber producers, tau saghyz and krym saghyz.

In 1930 S. L. Zaretski discovered *Scorzonera tau saghyz* (Lip-schitz and Bosse) in the Kara-Tau Mountains of central Asia (167). The root of this plant contained 5-30% of rubber (dry wt. basis) in the form of a latex. As a commercial rubber-bearing plant, however, it proved disappointing because of its great susceptibility to insect and fungus attacks. Seed production was also poor, making large-scale planting impractical.

The following year *Taraxacum kok saghyz* (Rodin) was discovered by a Soviet expedition in the Tien Shan Mountains of the

Kazakh S.S.R., near the border of China (167, 219). The root of this plant contains a lower rubber concentration than that found in tau saghyz, but its superior growth, propagation, hardiness and seed production characteristics established it as a more practical economic plant.

Krym saghyz (*Taraxacum megalorhizon* Hand.-Mazz.) is a Crimean dandelion whose roots contain 1–2% rubber after one year and 5–8% rubber after two years. However, the difficulties involved in carrying krym saghyz over the critical winter period have rendered it economically unimportant up to the present time.

The literature on kok saghyz has been reviewed in this journal (115).

Amount of Rubber Produced

In 1938, 170,000 acres of kok saghyz were grown in the Soviet Union. An expansion program called for an increase to 2,500,000 acres by 1941 (110), and in June, 1941, 2,000,000 acres were actually in production (50).

Estimates of yield vary greatly. Brandes (50) has estimated a production of 3,000 pounds of raw root and 30–60 pounds of rubber per acre. This estimate would, if correct, suggest that the U.S.S.R. obtained a minimum of 30,000 tons of rubber from their 1941 plantings. Kolachov (110) claims a production of 4,500–5,500 pounds of fresh root and 150–220 pounds of rubber per acre. In tests at Kew (14), kok saghyz yielded 65–100 pounds of rubber per acre, while in New Zealand others (227) report 5–279 pounds of rubber per acre. Dietrich (59) cites the production in the Ukraine as of the order of 90–135 pounds of rubber per acre, and Lysenko (126) estimates the yield of raw root at 2,200–3,100 pounds per acre. On the basis of experiments in the United States, Whaley concludes (219) that it may be possible to produce 400 pounds of rubber per acre from kok saghyz. Detailed data on the wartime production of rubber from 600 acres of kok saghyz grown in the United States can be found in the report of Eskew (67).

Estimates of the rubber concentration in kok saghyz roots also vary greatly. This is undoubtedly in part a reflection of the great variability in rubber yield between various individuals and strains of the plant. It has, for example, been reported (219) that plants grown from one lot of seed may vary in rubber content from a few tenths of one per cent to 16–18%. The workers at Kew have re-

ported rubber concentrations of 2-17% on a dry-weight basis. Bobkov (41) found 1.65% of rubber in fresh roots, while Brandes (50) estimates 2-2.5% of rubber on a fresh-weight and 6-7% of rubber on a dry-weight basis at the end of one year. Kolachov (110) estimates that rubber content may reach 26% on a dry-weight basis, and this concentration is said to have been attained in Canadian grown plants (219). Reichert (182) found an average of 12% rubber in air-dry roots grown in the Kursk region. Others (227) report average yields of 0.4-2.4% on a fresh-weight basis. Ignatiev (96) estimates that rubber makes up 4-7% of the dry weight of the krym saghyz root.

Extensive work (65) has indicated that a rubber concentration of 4-5% after one season and of 12% after two seasons may be expected in the Kazan region.

Anatomy and Biology

Kok saghyz is an herbaceous perennial, forming a rosette of 20 to many leaves and several to many inflorescences. The rubber is contained mainly in the thickened tap root and is in the form of latex contained in latex vessels of the secondary phloem. The secondary phloem is made up of concentric bands of vascular tissue separated by parenchymatous cells (20). Within each band are the latex vessels, intimately associated with the sieve tubes. The latex vessels of one band are joined by numerous anastomoses, while there is little or no connection between the vessels of different rings, a situation paralleling that described above for *Hevea*. The leaves contain rubber both in latex vessels and in parenchymatous cells. Rudenskaya (185) has shown that, other things being equal, the rubber yield of a strain of kok saghyz is proportional to the total vessel area contained in a root cross section. This is not, however, the only factor influencing productivity of various strains. Rubber accumulation commences in the seedling (28) and continues through the second year of growth. During the second year, however, the older tissues of the root are progressively sloughed off and their rubber destroyed by microorganisms, so that only relatively small increases in total rubber per plant are obtained in the latter part of the second year (132).

Kok saghyz may be planted in the fall and harvested approximately 12 months later, or it may be planted in the spring and har-

vested during the next year. In either case the plant blooms in midsummer and may then undergo a summer, drought-induced, dormancy from which it revives in the fall. It subsequently undergoes a winter, cold-induced, dormancy, from which it emerges the following spring. Drobkov (65) concludes that maximal yields and optimum land utilization are achieved by planting in the spring and harvesting in the spring of the second year. The normal $2n$ chromosome number of kok saghyz is 16 (217), macrosporogenesis and fertilization are normal and apomixis is unknown. Tetraploid plants are more vigorous than the diploids (111) and offer considerable advantage over the diploid in seed crop and in plant size, although rubber percentage remains unaffected (157). Normal plants are self-sterile and cross-fertile in summer, but much more self-fertility is manifested in late fall and winter (217). Seeds are very small and require preconditioning or vernalization procedures to ensure good germination (50, 119, 126). Propagation may also be conveniently carried out by means of short root cuttings (130, 156).

Soil and Climatic Requirements

According to Altukhov (12), kok saghyz is very easily acclimatized to new surroundings, but produces its highest yields on chernozem soils, especially those high in humus, N and P. It will, however, produce appreciable quantities of rubber on podzols, swampy lowlands and peat soils. Others (49) report that an acid Beltsville soil (pH 5.8) is better than an alkaline Oregon soil (pH 9.05), although a good crop of kok saghyz may be grown on the latter. Kolachov (110) concludes that kok saghyz does best with about 20 inches of rainfall annually, with much rainfall during April and May. Temperature requirements of the plant are not known in detail, but crops have been produced as far north as Archangel. Growth is, however, poor in warmer climates.

It has been shown that the form of N supplied to kok saghyz in pot culture greatly influences the total yield and rubber content of the roots (127). In general, high nitrate leads to the production of large quantities of organic matter, whereas high ammonium leads to high rubber concentrations. The best over-all yield is produced by a judicious mixture of ammonium and nitrate nitrogen in the substrate. Other investigators (60) claim that optimum yields of rubber are obtained if nitrogen is applied lightly at first and in-

creased toward the end of the vegetative period. They also state that sucrose and inulin contents of the root rise with increased nitrogen, and that increases in the N:K ratio raise the reducing sugar-sucrose ratio in the leaves.

Brandes (50), on the other hand, states that heavy nitrogen is detrimental to rubber production, and advocates light applications of a complete fertilizer. Kalinkevich (101) feels that heavy applications of nitrogen during the last one to one and one-half months of growth (after fruiting) favor leaf expansion and protein synthesis at the expense of the root, and decrease the quality of the rubber. He claims that the highest yields of rubber are obtained when nitrogen is maintained at a high level before fruiting and lowered thereafter. Low nitrogen (or high potassium) levels toward the end of the growth period favor sugar storage in the roots at the expense of the leaves. The N:K ratio is also said to influence the type of sugar present, a high ratio favoring reducing sugars, and a low ratio favoring sucrose and higher sugars. Klechetov (105) claims to have shown that tau saghyz fixes atmospheric N with the aid of a mycorrhizal fungus, and that the plant can under his conditions develop more or less normally in a nutrient solution containing no fixed nitrogen. This unusual finding is yet to be confirmed.

The effect of phosphorus addition is apparently dependent on the moisture level in the soil. Neuman (158) reports that in humid years, high P increases the elasticity and viscosity of rubber in the plant, but that the opposite effect occurs during the dry seasons. According to Brandes (51), addition of phosphate increases the quantity of rubber produced in wet years, but not in dry years. Mikhailov (147) claims that high phosphate levels during the early growth of the plant increase photosynthetic ability, accumulation of carbohydrates, synthesis of proteins, storage of inorganic and organic phosphates and growth. The major element nutrition of kok saghyz has also been investigated in pot cultures (145).

Drobkov (63) has reported that radium salts supplied in low concentrations (10^{-8}) to solutions containing B and Mn increased the yield of roots and of rubber in kok saghyz by 32-50%. Rare earths produced a similar effect but increased rubber yields to a much greater extent (72-101%). These unexpected results have not as yet been confirmed.

Light and Temperature Relations

In kok saghyz plants bearing floral primordia and subjected to photoperiods of 8, 10, 12, 14, 16 and 18 hours daily, flowering occurred in all sets, but the 8- and 10-hour photoperiod series lagged somewhat behind the others (49). In a second experiment, plants were grown from seed and exposed for their entire life cycle to one of the above photoperiods. Again all flowered, with the two shortest photoperiods lagging behind the others. It may be said, therefore, that kok saghyz is photoperiodically indeterminate. The importance of this lies in the fact that flowering plants have been found to contain 40-60% more rubber than non-flowering plants, as well as more inulin and total carbohydrates (133).

Mazanko (136) reports that greater rubber deposition occurs in tau saghyz if the above-ground organs are shaded or removed at a particular stage of growth. Others (159) investigated the summer "rest" period in kok saghyz, during which the leaves are lost and the plant becomes outwardly dormant. This dormancy may recur in the same season if drought occurs after growth resumption in the fall. The dormancy may be prevented entirely by shading of the tops. Smolin (189) similarly found that the summer dormancy of tau saghyz, presumably due to drought, could be prevented by removal of the older leaves. He feels that this is due to the removal of "physically older tissue" in addition to the stimulatory effect of wound hormones, formed during defoliation, on general metabolism. Novikov *et al.* (165) have shown that with tau saghyz active rubber accumulation takes place up to temperatures at least as high as 28° C., while rubber accumulation slows down greatly below 10° C. The same authors found rubber accumulation to depend intimately on light intensity and to decrease with decreases in this factor. Milovidov (148) found that reduction of daylight to 50% of its normal intensity decreased dry-weight deposition in *Asclepias cornuti* by 19% and in kok saghyz by 43%. The decrease in rubber deposition was greater than that in carbohydrate accumulation.

Others (49), investigating the temperature relations of kok saghyz, concluded that cold-temperature treatments, when applied in the early stages of the plant's development, stimulate flowering. When this cold treatment is applied later in the life history of the plant, it is not so effective.

General Biochemistry of Kok Saghyz

Kok saghyz plants have been shown to have two distinct metabolic periods (162, 163). The first is characterized by intense leaf growth and carbon assimilation, little carbon storage and much protein formation in the leaves. As the plant matures, the second metabolic period sets in. During this stage protein synthesis declines, storage of carbon and non-protein nitrogen compounds increases, and rubber formation and storage also set in. Resins remain fairly constant through the entire growth period and are, therefore, considered a general metabolic by-product. With increasing age of the leaves, their photosynthetic ability declines sharply. At the same time, translocation of carbohydrates from top to roots occurs, greatly increasing the dry weight of the root late in the vegetative period. In general, rubber accumulation in the root occurs after a high carbohydrate level has been attained. The same workers found that the ash percentage decreases with growth in both leaves and roots. The sugar content of the leaf may reach a level of 11.5% of the total dry weight of that organ, whereas inulin in the root may surpass 50% of the dry weight. The rubber percentage rose during growth from about 1% to about 8%, whereas resins declined from 3.4% to about 1.7%. Dietrich (59) also found that kok saghyz roots may be more than 50% inulin.

Blokhintseva (28, 29, 30) detected rubber in kok saghyz seedlings the first day after germination, and found a steady rise in rubber percentage during subsequent growth. By microchemical methods she showed a gradual decline in resin per cent with increasing age of the plant, and thus a gradual increase in the rubber : resin ratio.

Drobkov (64) showed that much inulin is formed during the summer and fall of the first year, followed by a rise in rubber and drop in inulin during the late winter as well as the early spring of the next year. He presumed that inulin is in some way the precursor of rubber in the plant, just as starch is in *Hevea*. Rapid increases in rubber in kok saghyz plants in the field during the fall are associated with a corresponding utilization of inulin and other sugars (65).

Kalinkevich (102) found a shift of carbohydrates from leaves to reproductive organs during flowering, followed by translocation to the roots after fruiting.

Filin (69) showed that the latex concentration in krym saghyz

increases with increasing depth of the root. Translocation of latex from the lower to the upper part of the root may be induced by copious watering or by removal of the tops before digging. The translocation is thought to result from a latex flow in the intact root induced by altered turgor relations. These procedures are advocated as expedients for avoiding loss of rubber during harvesting, through failure to gather the deep portions of the root. Others (180) found that in kok saghyz the rubber concentration is low in the upper part of the root, reaches a maximum 18–25 cm. below the ground surface, and then decreases again.

Earlier suggestions that rubber is formed in the leaves and translocated to the roots of tau saghyz (166) have been shown, in a series of very elegant grafting experiments, to be incorrect (177). This disproof made use of the fact that latex particles of tau, krym, and kok saghyz may be readily differentiated from one another microscopically. In grafts of krym on kok saghyz perfect vascular and lactiferous union was made, but no interchange of latex particles between scion and stock could be detected. The same was found in grafts between various species of *Scorzonera*. Latex could be artificially transfused from stock to scion, or *vice versa*, by tapping one member of the union so that latex would flow across the graft (179).

The physiology of rubber formation in tau saghyz has also been studied (178), showing that rubber is synthesized by isolated roots and by etiolated plants, provided carbohydrates are supplied. That stored carbohydrate may also be converted to rubber by the excised root is indicated by work (203) in which kok saghyz roots were cut into 2–3-mm. sections, and the sections stored in glass jars for several months. At the end of this time the rubber content of the root segments had increased, while the inulin content had decreased. This finding has been confirmed by others (25, 190) who have also shown that rubber formation proceeds actively in stored live roots, provided the roots are allowed to wilt by a loss of 10–12% of water first. This previous wilting is essential to rubber formation in storage. Storage of dead or air-dry roots resulted in no change or in losses of rubber. Synthesis of rubber by kok saghyz roots in storage has also been observed elsewhere (142).

The interesting observation has been reported that excised kok saghyz roots from which latex had been partially removed by

tapping showed an increased rate of oxygen uptake and a decreased respiratory quotient as compared with excised roots from which latex flow had been prevented by cutting with a hot knife (161). It will be of interest to determine whether the increased respiratory activity has to do with replacement of the latex drained from the tissue. Grebinski (80) has found an inverse relation between activity of peroxidase and catalase and rubber content of the plant. A direct relation between a particular enzyme and rubber accumulation has not yet been demonstrated.

It has been found that rubber in kok saghyz roots remained constant after 45 days of storage at 0–5° C., or 30 days at –12° C., but that it decreased after 10 days at –18° C. (97). Others noted that drying the roots to about 20% moisture by exposure to the sun does not affect the quality of the rubber. There was, however, some decrease in the quantity of hydrocarbon during storage (160).

Function of Rubber

Rubber does not appear to be utilizable as a reserve food by kok saghyz. Lebedev (118), for example, showed that rubber did not decrease in kok saghyz plants kept in darkness for almost six weeks. In a critical review of the evidence concerning the rôle of rubber by kok and other saghyzes, Prokofiev (176) has come to the conclusion that rubber once laid down can not again be mobilized. Data of Mazanko (138, 139) which tended to suggest the contrary are shown by Prokofiev to be inconsistent and probably unsound. Disappearance of rubber from the root cortex of kok saghyz during the second year's growth is attributed (132) to microorganisms which attack the older root tissues and gradually destroy them.

GUAYULE

Introduction

Guayule (*Parthenium argentatum* Gray) is native to the Chihuahuan desert of northern Mexico and to the Big Bend region of Texas where it is found at elevations of 4,000 to 7,000 feet in areas which normally receive 7 to 14 inches of rain per year. It is capable of bearing extremes of heat in summer and temperatures as low as perhaps 5° F. (149) in winter. Although guayule was known by pre-Columbian Indians to contain rubber, the plant was first botanically discovered by J. M. Bigelow in 1852 during the Mexican boundary survey, and was turned over by him to Asa Gray for

description. With development of methods for commercial extraction of rubber, production of rubber from native wild plants in Mexico started in 1902, and the first commercial shipment reached New York in 1904. In 1910 approximately 9,000 tons of guayule rubber were exported from Mexico, this amount making up about one-fifth of the total U. S. rubber consumption (54). Production of guayule rubber from the wild shrub continues to the present day. Beginning in 1910 the Intercontinental Rubber Company undertook extensive research looking toward cultivation of guayule as a commercial crop. Selection of high-yielding strains was carried on by W. B. McCallum (140), and these strains are the ones which were planted during the rubber emergency. Experimental plantings were made by the Intercontinental Rubber Company in various sections of southwestern United States, and it was established that guayule could be grown in regions of California, Arizona, New Mexico and Texas. Headquarters of the experimental guayule work was established at Salinas, Calif., and extensive plantings were made in the Salinas valley. In March, 1942, the entire operations of the Intercontinental Rubber Company within the continental United States were taken over by the United States Government. Approximately 32,000 acres of government-owned guayule were placed under cultivation in the United States. A thorough survey of all work on rubber production from guayule in the United States up to and including 1944 is available in the form of a House report (168), and there are two bibliographies of the work up to 1942 (26, 155). Recent agronomic advances in the production of guayule have also been summarized (93).

Guayule cultivation has been carried out on a small scale in Italy, Lybia, Czechoslovakia and Australia, and test plantings have been established in Chile, Argentina and Uruguay (170). Large-scale plantings have also been made in Mexico (170) and in the Soviet Union (77, 78).

The genus *Parthenium* contains numerous species in addition to *argentatum*, notably *P. incanum*, the mariola, which is coextensive with guayule. *P. incanum*, as all of the species other than *P. argentatum*, contains negligible amounts of rubber.

Anatomy and Morphology of Rubber Production

The anatomy and morphology of the guayule and its rubber production have been reviewed (2, 18, 19, 120), and there are also

summaries of work on the reproduction of the plant (66, 173). Rubber is found in all organs of the plant, but the amount in the leaves is relatively low, and commercially important quantities are found only in the bark of the stems and roots. In the young actively growing stem or root rubber first appears in significant quantities in the cells lining the resin canals of the cortex, and to a lesser extent in the other parenchymatous cells of the primary cortex and pith. If environmental conditions favor rubber deposition (see below), rubber next appears in the parenchymatous cells of the secondary cortex and the vascular rays. Since rubber is so largely confined to the cortex and phloem, the content of rubber in any plant is a function of the bark-wood ratio, and it has been indicated that high-yielding strains may have more phloem and less xylem than low-yielding strains (18). Rubber accumulation in older plants follows the pattern of that in the young plant (19). Tissues of the current season's growth contain less rubber during spring and summer than the corresponding tissues of older growth. This new tissue then fills up with rubber as environmental conditions become favorable to rubber accumulation during fall and winter. Whether or not rubber continues to accumulate in any one cell during more than one season is known only in the case of the cells of the pith. In these cells the present authors have found that rubber is laid down during the first season and remains constant thereafter. The pith of new growth on a mature plant possesses the same concentration of rubber as the pith of two- to three-year-old wood. This indicates that any cell may perhaps not increase in rubber concentration after the first season of rubber deposition.

It should be stressed that guayule contains no organized latex vessels. The resin canals, which are abundant in the cortex, appear to contain only resin.

Lloyd (120) originally thought that the rubber of guayule is present in the parenchymatous cells as particles of coagulated, solid, rubber. Later, however (121), he indicated that rubber may be present as a suspension or latex just as in lactiferous plants, and there is now general agreement on this score. The fact that no latex flow results when a guayule stem is cut is due, then, to the lack of latex vessels rather than to a lack of latex. The usual method for recovery of rubber from guayule depends on coagulation of the rubber before extraction, and such coagulation is in general

achieved by allowing the plants to dry before grinding. If, however, coagulation is avoided, the rubber may be recovered as a latex (192). Moshkina (153) claims that, in addition to rubber, resins and terpenes are present in the parenchymatous cells of the cortex, and that the rubber is in essence dissolved in the terpene. This view is not supported by other work (82) indicating that the bulk of the terpenes is present in the leaves rather than in the bark of the guayule. Similarly the principal portion of the resin is present in the resin canals of the bark (120).

Reproduction of guayule is effected by seeds, produced either by sexual reproduction or apomictically. The guayule forms found in nature form a polyploid series in which the chromosome numbers 36, 54, 72 and 108 are commonly represented. Sexual reproduction is characteristic of the 36-chromosome types, while apomixis dominates in the 72-chromosome types, the latter including the principal economically valuable forms. Vegetative propagation of the plant is readily accomplished by cuttings (164).

Amount of Rubber Accumulated

Guayule would appear to be, together with kok and tau saghyz, among the most energetic rubber-accumulating species. Concentrations of rubber of 16% and over on a dry-weight basis of the plant have frequently been recorded, and individual old plants may contain as high as 22% of pure rubber hydrocarbon (141). Under cultivation, however, plants set out in the field in spring contain not more than 2-5% rubber after one year and 6-12% after two years. Yields of rubber per acre vary greatly, depending on soil, water and climatic conditions. Favorable conditions on irrigated land may be expected to result in as much as 240 pounds of rubber per acre after one year, and a yield of 900 pounds per acre after two years (168); Hildreth has reported (93) yields of 1,200-1,500 pounds of rubber per acre from direct seed field plantings less than two years old. Plants allowed to remain in the field until growth has essentially ceased (5-10 years) may contain as much as 2,400 pounds of rubber per acre (141), or even 2,700 pounds (168). In the Soviet Union smaller yields have been obtained. It is reported that the best regions for guayule cultivation are in lower Azerbaijan where yields of 670 pounds of rubber per acre may be obtained in four years (4).

Environmental Factors Affecting Rubber Formation

Temperature. Accumulation of rubber by guayule is, in a sense, cyclical (193). Little accumulation ordinarily takes place during spring and summer, but beginning in early fall rapid accumulation of rubber commences and continues through the winter. With the onset of the spring growing season rubber accumulation slackens, to be resumed actively the following fall. This periodic fluctuation in intensity of rubber accumulation is in part a response to seasonal temperature changes (43). Guayule plants grown under conditions of abundant available water and high fertility level were maintained under various controlled temperature conditions. It was shown that when the temperature was kept at 80° F. day and night, rubber accumulation was slow and no increase in rubber percentage resulted. If the night temperatures were lowered to 50, 45, 40 or 35° F., rapid increases in rubber concentration took place, the optimum temperature being 40–45° F. Temperatures below freezing are probably only slightly, if at all, effective in inducing rubber formation. Exposures of plants to low night temperatures for less than 10 hours were found to be ineffective under laboratory conditions, while exposures of 16 hours were highly effective (46). Low day temperatures (40–50° F.) combined with low night temperatures did not result in rubber accumulation, but a day temperature of 65° F. appeared to be as favorable as one of 80° F. when combined with 40–50° F. night temperature (46). Night temperatures low enough to initiate rubber accumulation also brought about cessation of flowering and of shoot elongation. The same temperatures did not, however, interfere with accumulation of dry matter, since plants subjected to cold nights increased in dry weight as rapidly as plants subjected to warmer nights over a period of several months (43).

Plants which had been subjected to low night temperatures for months promptly diminished their rate of rubber accumulation when returned to the high night temperature. Such low-temperature-treated plants were found, however, to be more effective accumulators of rubber at high temperature than were similar non-cold-treated plants, indicating that a sort of low-temperature "induction" may occur with respect to the rubber-forming process (43).

Water. It has been known since Lloyd's early investigations (120) that irrigated guayule plants tend to have lower concentra-

tions of rubber than non-irrigated plants. This was found to be due, in part at least, to a small phloem-xylem ratio in the plants under low water stress. The observation of Lloyd has been abundantly confirmed, both under laboratory conditions (45, 175) and in the field (95, 104). The interaction between temperature and water stress as factors affecting rubber accumulation has also been investigated (45). Under conditions of high temperature, high water stress caused increases in rubber concentrations above the amounts found in plants subjected to low water stress. The increases in rubber accumulation were smaller, however, than those attained as a result of low night temperature treatment. Smaller effects of water stress on rubber accumulation were noted under low night temperatures. These results are in accordance with field observations which have shown that the period of rubber accumulation may be prolonged into spring or initiated earlier in fall by subjecting plants to water stress. Total yields of rubber per plant from water-stressed plants are smaller under high fertility conditions than yields from well watered plants, owing to the smaller total size of the plants subject to moisture stress (45).

Others (104) have also shown that moisture stress results in increased rate of rubber deposition in guayule, both under the temperature conditions of summer and fall and under the cooler conditions of winter. In their experiment only concentration of rubber was affected, total rubber per acre remaining more or less constant. In studies of the influence of moisture stress on rubber deposition on field-grown plants, the rubber percentage was increased by moisture stress under all conditions (95). In a sandy loam, total rubber per acre was highest with high moisture levels. In a silty clay loam, on the contrary, the highest rubber per acre occurred in the series with the highest moisture stress. The anatomical effects of moisture stress have also been studied (2).

Nutrition. The relation between nutrition and growth and rubber accumulation in guayule has been investigated (44, 151). In general, both growth and rubber accumulation were decreased with decreases in available N or P. Abundant nitrogen also appears essential for good seed production (222). Variation of sulfate over a wide range was found to be without influence. Variations of K, Ca and Mg similarly had but little influence on growth or rubber accumulation over a wide range of concentrations. Even though

plants grown continuously on low N or P accumulated rubber to a lesser extent than plants receiving adequate amounts of these materials, actively growing plants maintained at high temperatures and at a high fertility level may be caused to show a sudden increase in rubber concentration by restriction of available N or P (45). Increased yields of rubber under field conditions have been reported (117) to result from application of phosphate.

Plants in gravel culture accumulated less rubber when supplied with ammonium as a source of N than when supplied with nitrate (44). Mixtures of nitrate and ammonium N were intermediate in this respect. The conditions of the experiment precluded any extensive conversion of ammonium N to nitrate N, as would take place under field conditions. Ammonium nitrogen would of itself, however, appear to be unfavorable to rubber accumulation in guayule. Just the reverse situation has been reported (127) for kok saghyz, where maximum rubber accumulation results from the use of ammonium nitrogen, and smaller yields follow nitrate application.

Others (150) have shown that growth and rubber formation of guayule are quite sensitive to boron deficiency, the optimum boron concentration for plants in gravel culture being 0.1–2 p.p.m. in the nutrient solution; higher boron concentrations resulted in depressed growth and in lowered rubber concentrations.

Light Intensity. Results obtained from indicator plots have shown that, other conditions being equal, rubber accumulation and rubber yields are greater in the interior regions, such as the Coachella Valley of California and the Salt River Valley of Arizona, than in the coastal areas, such as the Salinas Valley of California. This may be, at least in part, a response to light intensity. An experiment conducted in the Coachella Valley showed that if plants were shaded so as to reduce light intensity to one-half, then dry weight and rubber accumulation were both decreased by approximately one-half (45). Plants shaded so as to decrease light intensity to one-fourth resulted in a diminution of dry weight and rubber accumulation to one-fourth of the level attained in full light. Similar results were obtained with guayule grown in Maryland (151). The latter authors showed, in addition, that low light intensity is a more severe limiting factor under conditions of high soil fertility than under conditions of low soil fertility. Guayule is apparently particularly adapted to regions of high light intensity.

A similar effect of decreased light intensity in decreasing rubber accumulation has been noted for *Asclepias* and krym saghyz (148). In this connection it may be remarked that it is difficult or impossible to grow guayule plants in the greenhouse so that they resemble plants grown out of doors in the summertime. Greenhouse-grown plants have a strong tendency to assume a spindly etiolated character.

Effect of Leaves on Rubber Accumulation

Removal of leaves during the fall, or principal rubber-accumulating season, results in complete or nearly complete cessation of rubber accumulation by the woody portions of the plant (45, 193). This is so even though the woody portions contain large amounts of carbohydrate reserve material which are gradually utilized in the maintenance of the defoliated plant. Plants maintained in darkness or under reduced light intensity, similarly fail to transform reserve materials into rubber. These facts suggest that compounds essential for rubber synthesis in the bark may be synthesized in the leaves and transported to the bark where they are utilized without intervening storage as carbohydrates. It may be noted that under field conditions inulin (143, 86), the principal reserve carbohydrate, is normally accumulated simultaneously with the accumulation of rubber (cf. 19), indicating again that carbohydrate and rubber synthesis are separate mechanisms, rather than links in a chain.

Other Hydrocarbon Derivatives

Guayule forms appreciable amounts of hydrocarbons other than rubber, especially resins and oils. The constitution of these materials was first studied by Alexander (3), who demonstrated that guayule oil contains much α -pinene. Fresh plants contain the oil principally in the leaves and to the extent of 0.2–0.4% on a fresh-weight basis. The presence of aldehydes as well as α -pinene in the oil has also been demonstrated (153). Quantitative study of the oil has shown the following composition (82):

TABLE 2
COMPOSITION OF GUAYULE LEAF OIL

α -pinene	60%	Phellandrol	4%
β -pinene	2%	Sesquiterpene with azulene	
Dipentene	9%	nucleus	3%
Cadinene	8%	Guayene-like sesquiterpene	2%
Other terpenes	6%	Terpene ketone	2%
Elemol-like sesquiterpene			
alcohol	4%		

The resins consist in part of the cinnamic acid ester of the sesquiterpene alcohol, partheniol, and also contain free cinnamic acid (216). Free cinnamic acid has been isolated also from the medium in which guayule roots have been allowed to stand briefly (48). The cinnamic acid thus released from the root is in part responsible for the mutual growth inhibition by guayule which has been described (48). It has been noted (175), and confirmed by the present authors, that the concentration of volatile terpenes in leaves of guayule is greater during spring and summer and less during fall and winter, a periodicity the reverse of that of rubber formation. The magnitude of the volatile terpene formation during spring and summer is possibly very extensive, since considerable quantities of the more volatile components are lost to the atmosphere, a fact which is reflected in the typical odor of the guayule field. Prokofiev has suggested that in guayule a precursor common to terpenes and rubber is formed, and that under particular conditions this precursor is polymerized to terpenes and the other guayule oils, while under other conditions, such as those prevailing in fall and winter, the precursor is transformed to rubber.

Parthenium incanum, mariola, a species closely related to guayule, although it produces negligible amounts of rubber, does produce an abundance of essential oils. Apparently mariola possesses the biochemical systems necessary for terpene production but lacks the mechanism for switching oil synthesis to rubber synthesis.

Rubber Accumulation and Growth

Rapid increases in rubber concentration in guayule may be brought about by lowering the temperature, especially at night, by sudden restriction of available nitrogen or phosphorus, or by placing the plant under water stress. All these agencies also diminish the growth rate and change the growth habit of the plant, *i.e.*, decrease flowering, new leaf production and shoot elongation. Accumulation of dry weight is, however, affected to a lesser extent. It would seem that rubber accumulation occurs particularly under conditions favorable for assimilation but unfavorable for excessive vegetative growth. That the relation is a simple nutritive one is, however, unlikely in view of the fact pointed out above that even during vigorous vegetative growth large amounts of terpenes are synthesized, although in the form of essential oils rather than as rubber.

Function of Rubber

Spence and McCallum (193) have presented the view that rubber functions as a reserve food in guayule. Their evidence consisted of data which was believed to indicate losses of 10–15% in total rubber per plant during periods of vigorous growth either in the spring or after cutting back. Rubber losses were also observed in plants which were maintained in a defoliated state during periods of one month or more. Russian experiments with guayule, summarized by Prokofiev (175, 176), failed, however, to reveal utilization of rubber by plants under conditions of either mineral or carbohydrate starvation. The present authors have also failed to detect significant losses of rubber in plants kept defoliated for periods of one to six months, in plants held in darkness for one month, or in plants cut back and allowed to grow at the expense of their stored reserve. Neither the Russian experiment nor those of the present authors are directly comparable with those of Spence and McCallum, since the latter authors used plants of much higher rubber content (12–14%) than were available to the other workers. Traub (205) has also obtained data which indicate that even with plants relatively high in rubber concentration, rubber utilization is negligible. The conclusion may be safely drawn, however, that loss or utilization of rubber by the guayule plant, even under conditions of severe carbohydrate stress, is in the most extreme case still relatively small, and that rubber is at most a sluggishly available reserve food material. In this connection it should be pointed out that loss of rubber by the living plant does not necessarily indicate actual mobilization as a reserve food. It is conceivable that alterations, either of an enzymatic or of a non-enzymatic nature, might under appropriate circumstances alter the rubber so as to render it inert in the usual rubber determinations, without in the least converting the rubber into metabolizable units.

GOLDENROD

Introduction

Among the plants investigated by Thomas A. Edison in his systematic search for native American rubber-forming species, goldenrod has attracted the most attention and has been the subject of some investigation. Polhamus (169) has shown that the rubber of *Solidago* species is confined to the leaves and is not present in

significant amounts in stem or root. Rubber contents as high as 6.3% on the basis of leaf dry weight were found. Great differences in rubber content between various species and individuals of the same species were detected. Volkhovskaya (214) has summarized work done in the Soviet Union on the introduction of *Solidago* into cultivation. *S. sempervirens*, *leavenworthii*, *mexicana*, *altissima* and *petiolaris* were found to be best adapted for culture. Yields as high as 225 to 450 lbs. of rubber per acre were estimated for two-year-old plantings.

Anatomy and Kinetics of Rubber Production

The rubber of *Solidago* occurs in the form of particles suspended among the plastids in the cytoplasm of the palisade and spongy parenchyma cells of the leaf. In general, one particle occurs in each cell, but occasionally as many as five are found (184). This rubber appears to be coagulated rather than a cellular latex as in guayule. The leaves contain resin ducts lined by enlarged secretory cells. Resins occur in these ducts, but no rubber has been detected in them (184). Similar ducts are present in the pith and cortex of the young stem. The cells lining the ducts are the only stem elements which contain rubber. In older stems this tissue and the associated ducts are lost as a result of secondary thickening. Rubber is laid down in the young leaves, but accumulation continues up to the time of seed ripening and is particularly active during the period from flowering to seed maturity. Whether this is a reflection of the phase of plant development and age of the leaves or is an environmental effect due to changes in temperature or photoperiod is not known.

Biochemistry

Some aspects of the chemistry of *Solidago* have been investigated (113). Leaves of *S. leavenworthii* were found to contain 6–15% of resin and 0.3–1.5% of essential oil. The presence of α -pinene in the oil of *S. rigida* was indicated (71) but not conclusively demonstrated. Quercitrin and quercetin have been isolated from leaves of *S. leavenworthii* (81).

Goldenrod rubber is remarkable in that it possesses a much lower viscosity and hence is of lower molecular weight than that of *Hevea*, kok saghyz or guayule (79). The viscosity of goldenrod rubber, as extracted from the plant, is in fact closer to that of gutta percha

than to that of *Hevea* rubber. Goldenrod rubber for this reason yields vulcanizates of somewhat lower tensile strength and in general of lower quality than *Hevea*, kok saghyz or guayule. That the rubber of detached goldenrod leaves is rapidly destroyed by light has also been shown (174). This photo-destruction has been studied with the extracted rubber (79), and oxidation shown to take place in benzene solutions under the influence of irradiation. The reaction appears to be catalyzed by the chlorophyll and other pigments commonly associated with the impure rubber.

CRYPTOSTEGIA

Introduction

Cryptostegia grandiflora, a member of the Asclepiadaceae, is a shrub native to Africa and contains rubber in the form of a latex in latex vessels of the stems and leaves. The plant has been widely disseminated and grows wild as an escape from cultivation in tropical and semi-tropical areas of Mexico, Central and South America, and the Caribbean Islands. It can not tolerate frost, but is unassuming as to soil and grows well on a diversity of soil types (171). A summary of information available at the beginning of the rubber emergency has been prepared (98). This information suggested that since the rubber derived from *Cryptostegia* latex is of good quality and since the plant is fast-growing, it should be worthy of investigation as a possible source of rubber. Extensive investigation of *Cryptostegia* was carried out during the years 1942-1945, particularly by SHADA in Haiti, where a total of approximately 30,000 acres were planted, and in Mexico by a cooperative project of the U. S. and Mexican departments of agriculture. This work has established that *Cryptostegia* is not an economical producer of rubber, both because of the low yields obtained and because of the high labor cost involved in tapping the small stems of the shrub. Information of general importance relative to rubber formation in plants was, however, obtained by both projects.

Anatomy of Rubber Production

Cryptostegia grandiflora is a vigorous shrub of a semi-climbing habit. The morphology and anatomy of *Cryptostegia* have been reviewed by Artschwager (19a). In addition to ordinary stems, long, thin, vertical leaders or shoots, almost leafless and known as whips,

are formed. Large scale tapping of the plant for rubber has depended on tapping these whips. Rubber is contained in the stems exclusively in latex vessels which are present in the pith and in the bark. The vessels of the pith average about 25 microns in diameter and number 300-800 (57, 199). The bark contains somewhat smaller vessels, the number depending on the age and diameter of the stem. The vessels of the pith are laid down early in the life of the young stem and are filled with rubber-containing latex even before growth in length of the internode has ceased. These vessels do not increase in number during the subsequent life of the stem. The vessels of the bark are, however, continuously added to by secondary growth. Thus the older the stem the greater the proportion of its total rubber content which is contained in the bark. In young stems 3-4 mm. in diameter, it has been estimated that 75% of the rubber is in the vessels of the pith, while in a stem 17 mm. in diameter, 74% of the rubber is in the bark (223). In addition to the rubber of the stem, considerable quantities of the material are contained in the leaves. Slightly over 2% rubber has been detected in the leaves of *Cryptostegia grandiflora* in Florida and over 4% in leaves of the hybrid between *C. grandiflora* and the related *C. madagascariensis* (171). Individual plants with as high as 9.3% of leaf rubber on a dry weight basis were discovered. Two to four per cent rubber was found (94) in leaves from Cuba, Mexico, California, a range confirmed in the extensive study of the question by Stewart *et al.* (199). A portion of this leaf rubber is present in the form of latex contained in vessels associated with the midrib and larger veins of the leaf. Eighty to ninety per cent of the rubber occurs, however, as particles in the chlorophyll-bearing cells of the leaf, palisade, spongy parenchyma and guard cells (223, 224). The rubber particles, which are quite distinct from the chloroplasts, are 3-12 microns in diameter. They are found in large numbers in the parenchyma of older leaves, but are absent or nearly so from young leaves. They must, therefore, develop during development of the leaf.

Physiology of Latex Flow

Harvesting rubber from *Cryptostegia* has been based mainly on periodic tapping of the elongated nearly leafless stems, or whips, which are sent out in numbers by the plant and which may attain a length of 6-12 feet and a diameter of from a few mm. at the tip

to perhaps 2-3 cm. at the base. The latex is obtained by cutting the whip completely through at a point near the tip and collecting the latex which flows from the stump. At intervals a fresh cut is made in which a variable amount of stem is removed and the flow is renewed. The flow of latex continues for only 2-12 minutes after the tapping cut and then ceases, due in part to coagulation of the latex, in part possibly to diminution of the forces causing latex flow. A great number of experiments with varied tapping regimes have been carried out, a portion of which have been reported (199). In general, successive tapping leads to a rapid diminution in amount of latex and of rubber obtained per tapping, though the yield may be stabilized at a very low level. No evidence for any considerable regeneration of rubber in the latex after tapping could be found, in marked contrast to the rapid regeneration of rubber in *Hevea* noted above. Exhaustive tapping of whips over a period of months yielded a total of only two to three times as much rubber as was present in the initial whip, an increase which might be in part accounted for on the basis of increase in bark latex vessels during the tapping period. That any renewal of rubber within a mature vessel occurs is hence questionable. A striking dilution reaction was found to occur during the latex flow, the initial latex being approximately four times as concentrated as the final portion. Osmotic reconcentration of the latex after cessation of flow occurred within one to one and a half hours, that is, after this period a second tapping cut yielded latex which was more concentrated than the final portion of latex obtained from the first cut.

That the driving force of latex flow is osmotic in origin in *Cryptostegia*, as in *Hevea*, is indicated not only by the dilution and reconcentration reactions but also by the fact that latex flow is greatly influenced by the water balance of the plant. Thus drought or dry winds were found to cause striking decreases in latex flow, whereas conditions of low water stress favored copious flow (57, 199).

The length of stem involved in latex flow has been determined by the double tap method. For example, of two bleeding cuts, 80 inches apart, each yielded latex flows only slightly smaller than that obtained from a single tap. Cuts closer together resulted in decreased flow, the data showing that under the conditions used, half of the flow must have had its origin within 18.5 inches of the tapping

cut. This was confirmed in other experiments in which the stem was frozen by dry ice application and a tapping cut then made at varying distances from the block thus created. It was also shown that during the first few minutes of flow only a few inches of stem are involved, but that as the flow continues, more and more of the stem is involved. Even in the most extreme case, however, half of the flow appeared to originate within 27 inches of the tapping cut. It would appear, therefore, that a relatively small portion of the whip can be drained from a single tapping cut. That latex from one whip may not be transferred to and bled from a second has been shown by the fact that latex yield in a whip is independent of the number of other whips bled on the same plant.

Constitution of Latex and of Leaf Rubber

The general constitution of the latex of *Cryptostegia grandiflora* has been investigated by several workers (e.g., 31, 199). The latex may be coagulated by vigorous stirring and separated into a coagulum consisting essentially of the latex particles, and a serum which is rubber-free. The coagulum or particles consist of rubber, acetone-soluble resins of unknown nature, and protein, the rubber making up about 85% of the whole. The protein is presumably that bound to the rubber particles (188). The serum contains proteins, considerable amounts of KCl, compounds containing α -amino N, presumably amino acids, traces of citric and malic acid, and a large amount of material which is apparently a phenol (198, 199). The proteins include a highly active enzyme, peroxidase, and a less active laccase-like oxidase. The rubber-containing globules of the leaves of *Cryptostegia grandiflora* have been isolated by a method involving retting with *Clostridium roseum* (223). The cell walls are weakened by this treatment, and mechanical stirring results in liberation of the rubber globules. Boiling the suspension of globules with dilute NaOH results in creaming or rising to the surface of the particles. Analysis showed the globules to contain approximately 65% of rubber and 26% of acetone solubles. The acetone-solubles of whole *Cryptostegia* leaf rubber contain ursolic acid and higher paraffins of the sort generally contained in leaf waxes (221).

The globules of the leaves differ from the latex particles not only in over-all composition but also in the nature of the rubber. The leaf globule rubber is of low molecular weight, 10,000–16,000, and

is almost completely soluble in methyl ethyl ketone (94), a solvent for low molecular weight and degraded rubber, but in which high molecular weight rubber is insoluble (56). *Cryptostegia* latex rubber, on the other hand, like *Hevea* rubber, consists mainly of high molecular weight molecules and contains only a small portion of material soluble in methyl ethyl ketone (94). It is of interest to note that *Cryptostegia* leaf rubber resembles goldenrod leaf rubber not only in mode of occurrence but also in molecular size.

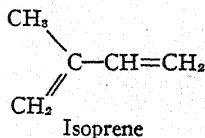
Yields of Rubber

Early estimates of rubber yields to be expected from *Cryptostegia* were based on the yield of leaf rubber from periodic trimming of the plant. Thus Dolley (62) has estimated that 400 lbs. of rubber per acre per year might be obtained in this way, while Jenkins (98) concludes that approximately 200 lbs. is all that should be expected. In any case, since the leaf rubber is of poor quality, the yield from this source is no longer of interest. Yields from whip bleeding are somewhat smaller than the theoretical yields from leaves. Thus in Mexico a maximum of 175 lbs. of rubber per acre per year could be obtained from two-year-old plantings (199). This yield was achieved only by use of tapping frequencies which would be impractical on a large scale, and they suggest that two-thirds of 175 lbs., or about 120 lbs., per acre is probably as much as can be hoped for from commercial *C. grandiflora* plantings at the present time. It has been estimated that as much as 200 lbs. of rubber per acre per year might be obtained by a modified whip bleeding procedure in which the rubber would be allowed to coagulate at the cut end of the whip, and the sections bearing these coagulated plugs of rubber would then be cut off and the rubber milled out mechanically (68).

BIOGENESIS OF RUBBER

Community of the Terpenes

Although essential oils, balsams, resins, camphors, carotinoids and rubber are differentiated by their physical properties and chemical structures, they can all be regarded as derived by polymerization of isoprene, C_5H_8 :



The isoprene derivatives, or terpenes, may be regarded as a chemical family, and the following table gives a brief summary of the relations existing between the several members of this family:

TERPENE CLASS	EMPIRICAL FORMULA	EXAMPLE OF HYDROCARBON	EXAMPLE OF OXIDATION PRODUCT	
Isoprene	C_5H_8			
Monoterpene	$C_{10}H_{16}$	Pinene	$C_{10}H_{16}O$	Camphor
Sesquiterpene	$C_{15}H_{24}$	Bisabolene	$C_{15}H_{24}OH$	Farnesol
Diterpene	$C_{20}H_{32}$	Camphorene	$C_{20}H_{32}OH$	Vitamin A
Triterpene	$C_{30}H_{48}$	Squalene	$C_{30}H_{48}OH$	Amyrin
Tetraterpene	$C_{40}H_{64}$	Carotenes	$C_{40}H_{56}O_2$	Xanthophylls
Polyterpene	$(C_5H_8)_n$	Rubber, gutta		

Synthesis of Various Terpenes by the Same or Related Plants

It may be said with considerable assurance that all plants possess the ability to form at least a few compounds of the terpene family, although in numerous species this is restricted to the carotinoids, phytol and sterols. The ability to form essential oils, *e.g.*, mono- and sesquiterpenes, appears scattered in families randomly distributed through the plant kingdom. Typical examples of terpene-forming species are *Mentha piperita*, the peppermint, and *Mentha spicata*, the spearmint (106). Here ability to form terpenes is restricted to the lower more volatile members (and carotinoids) and their derivatives. In other species, as in turpentine of the pines, a whole series of terpenes, sesquiterpenes and diterpenes occurs (106). Conversely, however, higher members of the terpene family may be formed by a particular species of plant without the occurrence of lower members. This is apparently the case in *Hevea*, where, aside from carotinoids, *etc.*, rubber is the only terpene formed in significant amounts. In other cases, however, formation of rubber and of other terpenes proceeds within the same plants. It has been pointed out above that in guayule, in addition to rubber, an oil, mainly α -pinene, is formed in large amounts. The data obtained by Prokofiev (175) suggest that oil formation may be antagonistic to rubber formation, since when rubber formation increased in rate, owing to favorable environmental conditions, oil synthesis decreased, and *vice versa*. A similar case has been studied more elegantly with two *Cryptostegia* species (1). *C. madagascariensis* produces a latex containing a large amount of a triterpene alcohol of the β -amyirin type. In crosses between *C. grandiflora* and *C. madagascariensis*, the formation of rubber *vs.* triterpinol was inherited as a simple

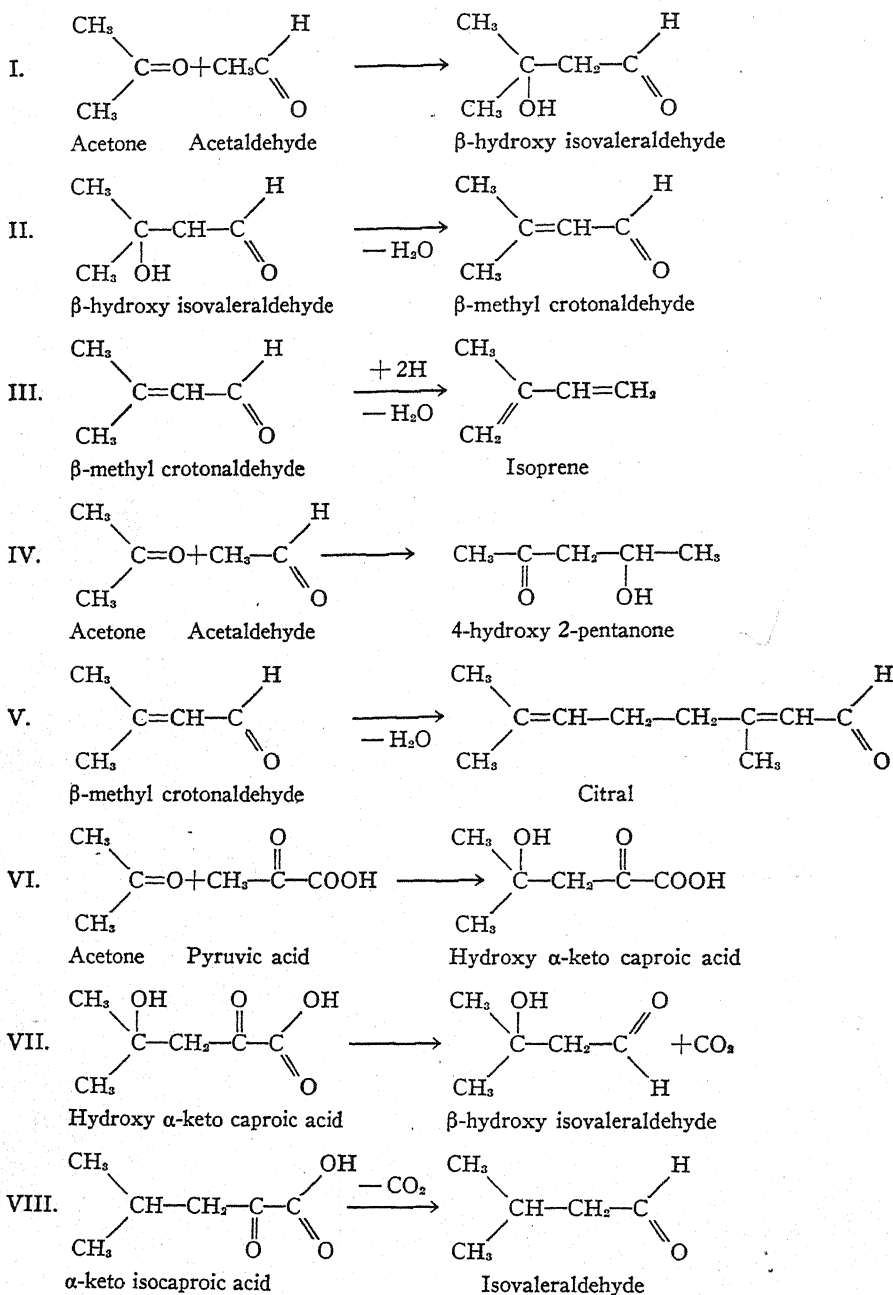


FIG. 2. A summary of chemical reactions having possible relation to the biosynthesis of rubber.

Mendelian character with rubber formation dominant. This result suggests most strongly that the two terpenes are polymerized from a common precursor and that in the presence of a particular enzyme, whose production is genetically controlled (22), this precursor goes to rubber; while in the absence of this enzyme but in the presence of another, triterpene is formed. That a similar condition may exist in other lactiferous plants is perhaps indicated by the wide occurrence of β -amyrin-like triterpene alcohols (or esters of such alcohols) in other latices, *e.g.*, kok saghyz (1), *Castilloa* (106), *Ficus* sp., chicle (106), *Euphorbia* sp. (218) and *Asclepias cornuti* (134).

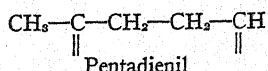
An important aspect of the view that the various terpenes arise from a common precursor is the supposition that the mode of polymerization of this precursor can be influenced by factors, possibly but not necessarily, of a protein, enzymic nature. The presence of specific enzymes would then result in the production of the specific isoprene polymer, whether rubber, gutta, triterpene, *etc.*

Anatomical Aspects of Terpene Formation

The relation of latex vessels to rubber accumulation is unclear. With but few exceptions, rubber is confined to latex vessels, whereas other terpenes are by and large produced in other tissues. Conversely, there are few, perhaps no cases, in which a latex does not contain either polyterpene (rubber or gutta) or triterpene or both (75, 210). The environment of the latex vessel or cell seems somehow to favor production of high molecular terpenes over lower polymers, and at the same time the existence of latex vessels would seem to go hand in hand with the production of highly polymerized terpenes. The carotinoids again form an exception, since they seem to occur in all plant cells.

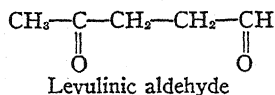
Possible Precursors of Terpene Formation

The exact chemical nature of the precursor from which rubber or other terpenes are synthesized remains a matter for speculation at the present time. Harries (85) has advanced the view that rubber is formed from pentoses through the intermediary of pentadienil:

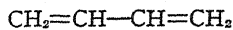
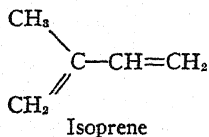


which should, according to him, condense immediately to rubber. The theory of Harries is supported by the fact that small amounts

of levulinic aldehyde are recovered among the decomposition products of ozonized rubber. Levulinic aldehyde is structurally related to the hypothetical pentadienil:



The hypothesis of Harries (see also 200), while it might appear to account for the synthesis of rubber, could not be fitted into a general scheme for the synthesis of other terpenes, such as those which contain an isopropyl side chain and in which the branched chain carbon skeleton of isoprene must be regarded as the unit. Others (*e.g.*, 21, 175) have suggested that isoprene itself is the material from which other terpenes are built up. This view would appear to be supported by a report (100) that latex contains enzymes which aid in the polymerization of butadiene and related compounds to rubber-like materials:



Butadiene

Ambros (13) has also reported experiments in which "enzymes" derived from latex were believed to have promoted the polymerization of isoprene to a rubber-like substance. Since the catalyst of Ambros was prepared by treatment of the whole latex with papain, in order to remove the rubber particles, it is doubtful that it was actually enzymatic in nature. Isoprene has never been detected in plant material, and its position in relation to rubber biogenesis is hence of the same doubtful nature as the position of formaldehyde in relation to the first product of photosynthesis.

Prokofiev (175) and others have suggested that isoprene may be formed from acetone and acetaldehyde through the intermediary of β -hydroxy isovaleraldehyde (I). This compound would be in turn dehydrated to β -methyl crotonaldehyde and reduced to isoprene (II and III). Condensation of acetone with acetaldehyde has been studied experimentally by Kuzin and Nevrajeva (116) who have shown that in the presence of glycine, condensation to yield β -hydroxy isovaleraldehyde actually occurs *in vitro*. Glycine appears to

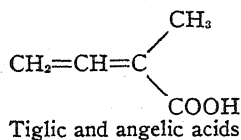
act as an agent modifying the course of the condensation which in the absence of the modifying agent yields mainly 4-hydroxy 2-pentanone (IV). Condensation of acetone and acetaldehyde has not, however, been demonstrated to take place either *in vivo* or under the influence of plant enzyme systems. Neither β -hydroxy isovaleraldehyde nor β -methyl crotonaldehyde has been certainly detected in nature. Isovaleraldehyde, on the other hand, is a common constituent of essential oils (218), and its oxidation product, isovaleric acid, is also commonly found. The scheme which Prokofiev has suggested as to the course of rubber synthesis, while straightforward and intriguing, has not yet been substantiated in any essential detail and can be regarded only as a suggestion for experimental work.

Kremers (114), in a study of terpene formation in peppermint, has also suggested that the first steps may consist of the condensation of acetone with acetaldehyde to form β -methyl crotonaldehyde, two molecules of which would then be condensed and dehydrated to the ten carbon aldehyde, citral (V), which is a common constituent of essential oils. Citral is regarded by Kremers as the basic terpene precursor, yielding other of the monoterpenes by oxidation, reduction and dehydration, combined with ring closure. Kremers regards isovaleraldehyde as a by-product, not in the main path of terpene synthesis, but a result of incidental reduction of β -methyl crotonaldehyde. The possibility that citral may also mark an intermediate in rubber synthesis is certainly not to be excluded, although this compound also is not commonly found in rubber-bearing plants in appreciable amounts.

Although it is quite conceivable that β -methyl crotonaldehyde might be built up, as suggested by Kremers and by Prokofiev, from the union of a C_3 with a C_2 compound, it is also possible that the C_5 skeleton might arise in nature by the degradation of compounds having a larger number of carbon atoms, as for example, by decarboxylation of the appropriate α -keto isocaproic acid. Thus α -keto isocaproic acid might be formed by condensation of acetone and pyruvic acid (VI). Decarboxylation of this keto acid would result in β -hydroxy isovaleraldehyde (VII). α -keto caproic acid itself is related to isoleucine, which might suggest a basis for the relationships which have been found between nitrogen metabolism and rubber formation, since the isocaproic acid might be decar-

boxylated to isovaleraldehyde (VIII). Nothing is as yet known concerning the genesis of the carbon skeleton of isoleucine.

The unsaturated five-carbon acid, tiglic, and its isomer, angelic acid, are common constituents of plant oils and possess the same



carbon skeleton as isoprene. The corresponding aldehydes have not been found in nature.

Still another possibility as to the basic precursor in the formation of terpenes is that it may be no simple C_5 compound, but that the C_5 units from which terpenes are built up are split off from larger molecules, the C_5 units being then immediately condensed to higher terpenes. In this case it might prove to be impossible to isolate a stable five-carbon precursor. A situation partly analogous to this possible one is that presented in levan formation by autolyzed cells of *Aerobacter levanicum* (92). Here sucrose is dissimilated to glucose and levan, the energy necessary for formation of the polysaccharide being supplied from that released by the hydrolysis of the sucrose.

The precursors of terpene formation can presumably be discovered by the application of straightforward biochemical methods, such as those applied to the biogenesis of amino acids (202), to the biogenesis of thiamin (47), or started in the case of the rubber of *Cryptostegia* (1). Until further basic experiments have been carried out it is impossible, however, to present any definite picture of the first steps in terpene biogenesis.

Mashtakov (132) has given evidence that the degree of polymerization of rubber in kok saghyz increases as the plants mature, and certain of his results are given in Table 3. Prokofiev (175) regards this increase in average molecular weight as being due to deposition of rubber of increasing molecular weight as the season progresses rather than to increases in the molecular weight of rubber molecules already present. It is clear in any case that physiological factors may influence the degree as well as the nature of polymerization.

TABLE 3
DEGREE OF POLYMERIZATION OF KOK SAGHYZ RUBBER AS A
FUNCTION OF AGE OF PLANTS
(Plants in 2nd year of growth. Leaves appeared on April 21)

Date	July 4	Aug. 3	Sept. 3	Oct. 4	Nov. 15
Av. molecular weight	60,000	100,000	136,000	170,000	250,000
Degree of polymerization	900	1,400	2,000	2,500	3,600

CONCLUSIONS

Anatomical Factors in Rubber Formation

With but few exceptions, *e.g.*, goldenrod, rubber is accumulated in the form of latex. This latex is produced and accumulated, in general, in specialized latex vessels, especially those vessels which occur in the bark; in a few cases, as in guayule, the production and accumulation take place within the cells of the parenchymatous tissues. In occasional cases the principal rubber accumulation may be in the leaves, as in goldenrod. By and large, however, rubber production by the latex system of the bark is most important from an economic standpoint. The production and accumulation of rubber differs, from an anatomical standpoint, from that of the related mono- and sesquiterpenes which are in general produced in cells or glands at or near the surface of leaves or bark or adjacent intercellular ducts or spaces.

Rubber and the other higher terpenes do not appear to be able to move out of the living cells in which they are synthesized, and no evidence for movement of rubber from cell to cell has been adduced. Rubber once laid down appears to remain *in situ* indefinitely. The lower terpenes, on the contrary, do move readily out of the cell in which they are produced, and frequently even escape to the outside air.

In general, it may be said that rubber deposition takes place most vigorously in mature tissues. Thus seedling guayule plants accumulate a smaller proportion of rubber than do older plants under identical conditions, while in kok saghyz rubber formation occurs principally in the mature root. The rubber content of latex from young rubber trees or from young parts of the tree is less than that of latex from mature trees (112).

Environmental Factors Influencing Rubber Formation

Temperature. It has been shown that low temperatures, particularly low night temperatures, favor the accumulation of rubber by guayule. These same temperature conditions are, however, unfavorable to rubber accumulation in tau saghyz and presumably to that of the tropical species, as *Hevea* and *Cryptostegia*. The high temperatures most favorable to rubber production in tau saghyz and presumably in the tropical species are conversely very unfavorable to rubber accumulation in guayule. No general rule can, therefore, be laid down concerning the rôle of temperature as a regulator of rubber production.

Nutrition. In *Hevea*, in kok saghyz and in guayule, high levels of nitrogen favor, with certain reservations, both growth and rubber formation; and nitrogen appears readily to become a limiting factor in rubber production. This is of interest from the standpoint that the rubber itself contains no nitrogen, so that the effect must be of an indirect nature and on the rubber-producing system rather than on the rubber *per se*. Phosphorus may also become a limiting factor in rubber production in all of the above mentioned species, while other deficiencies appear to be less important.

Light. In tau saghyz and in guayule, light intensity has been reported to be intimately related to rubber formation. Decrease in light intensity results in a proportionate decrease in rubber formation, even over relatively short periods of time. It would appear doubtful in the case of guayule that reserve carbohydrate can be converted to rubber, as indicated by the absence of rubber synthesis in defoliated plants, even in the presence of abundant reserve materials, as well as by the fact that rubber and reserve carbohydrates are accumulated simultaneously. In *Hevea* and kok saghyz, on the other hand, defoliated plants can continue to make rubber over short periods at the expense of stored carbohydrates. It would appear, therefore, that there is no general direct connection between photosynthesis and rubber accumulation, the case of guayule forming a possible exception. Since storage of rubber represents the investment of much chemical energy, it is only to be expected that there should be an over-all relation between assimilation and rubber accumulation over long periods. Thus defoliated plants of *Hevea* and kok saghyz also show decreased rubber accumulations as compared with normal leafy plants over long periods.

The Biochemistry of Rubber

The Rôle of Rubber. The suggestion that latex might serve to protect the plant against attacks from herbivorous animals, as snails (107), has been disproved by Tobler (204) as well as by numerous other observations, such as the fact that kok and tau saghyz roots may suffer severely from insect damage (108). Camels are also said to relish the rubber-bearing plant chondrilla (176). Other authors have urged that rubber may function as a reserve food (138, 139, 193). The bulk of the evidence available at the present time indicates, however, that rubber once deposited by the plant, in *Hevea*, in kok saghyz or in guayule, is thereafter either mobilized but little and relatively sluggishly or is perhaps not available at all as a reserve food. This conclusion is in agreement with that reached by earlier reviewers (75, 144, 176). That enzymes capable of bringing about rubber breakdown must exist is shown by the fact that rubber is attacked by many microorganisms (194, 226). Breakdown of rubber through the agency of enzymes from higher plants has, however, never been achieved. It would be of evident interest to determine whether such enzymes do occur in higher plants.

With the possible exception of a rôle as a secondary reserve food, no physiological function of rubber in the plant has as yet been found. It seems possible that this material is to be considered as an excretion product or a non-functional by-product of cellular metabolism. A similar view of the mono- and sesquiterpenes would seem all the more reasonable in view of the large amounts of these materials which are lost to the surrounding atmosphere and hence lost so far as further metabolic usefulness is concerned. It has been shown, for example, that one specimen of *Juniperus excelsa* may lose as much as 30 grams of essential oils per day to the surrounding atmosphere (112). Neither is any physiological function known for the triterpenes or their derivatives. Frey-Wyssling (75) considers it probable that a portion of the carotenoids (tetraterpene derivatives) may also represent functionless metabolic by-products.

Synthesis of Rubber. It would appear that in certain plants at least rubber is synthesized as an alternative to the formation of other less highly polymerized products. In guayule environmental factors appear to direct the metabolism either toward mono- and sesquiterpenes or, under other conditions, toward rubber. In *Cryptostegia* genetic factors determine whether rubber synthesis or synthesis of

a triterpene alcohol shall predominate. This evidence suggests that there may be a single terpene precursor which may be polymerized in various ways, depending on the conditions prevailing in the plant. This view is strengthened by the fact that all terpenes, from the simple monoterpenes to rubber and gutta, can be regarded chemically as derived from a single five-carbon branched chain skeleton, and compounds of the nature of isovaleric aldehyde, angelic aldehyde and tiglic aldehyde have been suggested as possibly related to the precursor. Numerous other possibilities may also be envisaged, however, and in the absence of any factual material derived from actual experiment, only guesses can be made as to the nature of this precursor of rubber.

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SUPERNUMERARY CHROMONEMAL REPRODUCTIONS: POLYTENE CHROMOSOMES, ENDOMITOSIS, MULTIPLE CHROMOSOME COMPLEXES, POLYSOMATY

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INTRODUCTION

It was decided, after some deliberation, that the title for this review should not be "Endomitosis", as originally intended, but rather the general one selected because it became increasingly apparent upon studying the literature that there is some difference of opinion as to the precise meaning of the term and as to which intranuclear phenomena might properly be considered endomitotic. It was felt also that there is a definite need for an integration of the principal contributions to our knowledge concerning all intranuclear phenomena which are either the cause or the result of chromonemal reproductions in excess of the ones associated with the ordinary processes of mitosis and meiosis.

Supernumerary chromonemal reproductions have been considered by many to be partly responsible for the increase in volume of individual chromosomes, especially the giant salivary gland chromosomes, where the reproduced elements remain associated. Chromosomes of this type are referred to as "polytene" or "polyvalent"¹. In other instances the supernumerary reproductions of the chromonemata are responsible for an increase in chromosome number when the reproduced elements, or so-called division products, separate from each other. Inasmuch as the supernumerary reproductions usually involve all the chromosomes of a somatic cell, the result is a multiple of the somatic number, and the fact that it is also a multiple of a more basic (haploid, monoploid, n or x) number is only incidental. Therefore, the terms "polysomaty" and "polysomatic", as first employed by Langlet (32), are more descriptive than the terms "polyploidy" and "polyploid" with reference to the situation where cells with the higher multiples of the somatic number of chromosomes are intermingled with normal somatic cells in the same organism.

¹ This term may also be applied in a different sense to trivalent and tetravalent associations of homologous chromosomes at first meiotic metaphase.

Many aberrations and deviations from normal nuclear processes have been considered to be responsible for the occurrence of multiples of the somatic chromosome number, and the term "endomitosis" in the original restricted sense employed by Geitler (17) describes only one method by which polysomatic cells may arise. However, in a more extended application the term "endomitosis" has been used by many to refer to any situation where supernumerary chromosomal reproductions occur within an intact nuclear membrane, regardless of whether these reproductions can be resolved into "endoprophase", "endometaphase", "endonephase" and "endotelophase", as these stages have been described by Geitler.

The biological interpretation of reproduction by division of a single unit of larger size into two like units of smaller size involves the concept that such division must always be preceded by internal growth and expansion. It has been indeed convenient to use the term "division" to describe cellular and chromosomal reproductions as they appear within the range of microscopic visibility. It becomes obviously necessary, however, to abandon this interpretation of reproduction when the units under consideration fall beyond the range of microscopic visibility and approach or lie within molecular dimensions. Reproduction of units within such a range must instead be interpreted to be the result of the accretion or of the accumulation and synthesis of substances like and alongside the original.

Sharp (59) distinguishes three levels of doubling of the chromosomal constituents: "(1) *elementary doubling*, in which the ultimate longitudinal constituents (protein chains?) of the chromosome become duplicated or multiplied probably through the formation of new ones close to the old ones by a process analogous to crystallization or polymerization; (2) *effective doubling*, in which the thread somehow reaches a stage at which a given agency such as X-rays may affect one longitudinal fraction and not another; (3) *visible doubling*, in which a thread appearing single under the microscope becomes double by a process that looks like real splitting". Therefore, since the terms "division", "splitting" and "cleavage" describe what is more apparent than real they will be avoided wherever possible, even at the risk of some inconvenience, in favor of such terms as "duplication", "multiplication" and "reproduction".

What Sharp has called "elementary doubling" has been the out-

growth of modern thinking with regard to the size, structure, arrangement and interrelationships of genes. Such modern ideas are perhaps best summarized by Demerec (14) who considers that a chromonema is composed of individual molecular units recognizable through their actions as genes. Recently Prokofieva-Belgovskaja (57) has used this concept as an aid in interpreting heterocyclic phenomena in binucleate cells in potato tubers. She has illustrated also a heteropycnotic difference between the right and left halves of a metaphase which she interprets to be made up of two complexes, one containing the original "mother" chromosomes and the other containing the reproduced copies or "daughter" chromosomes. In commenting on her paper, Muller (48) expresses some perplexity that the mother and daughter chromosomes should be separated into two groups in such a manner, and Lorz (40) adduces what he considers to be evidence in her illustration for polysomaty resulting from a double reproduction of the chromosomes. This evidence, if it has any validity, is inconsistent with such a grouping of mother and daughter chromosomes. This point will be elaborated further in a more detailed consideration of polysomaty which will form a considerable part of this review. Darlington (13) finds in the mitotic behavior of ring chromosomes a basis for the conclusion that "... there is an absolute distinction between a parent and a daughter thread in the reproduction of the chromosome".

POLYTENE CHROMOSOMES

The discovery of polytene chromosomes dates at least as far back as the year 1881 when Balbiani (3) described the nuclear contents of the salivary gland cells of *Chironomus* larvae. He noted the bands or discs and made the observation that the thicker bands could sometimes be resolved into component thinner ones. He noticed also that there were places along the body of the chromosome where it was divided into two halves and that the seriation, spacing and size of the bands in the one half corresponded to those in the other half. The literature contains very few accounts of further studies on the nature of the nuclear contents of the salivary glands of dipteran larvae in the half century following the publication of the observations of Balbiani. Alverdes (1) and Kostoff (31) studied what they called the "spireme" with the former interpreting the discs as gyres of a disintegrating chromonema and the latter

recognizing the true discoid nature of these markings and their possible association with gene loci. The final resolution of the spireme into a discrete haploid number of units which were identified as synapsed homologous chromosomes came three years later (20, 53). A large part of the work which followed these discoveries has been concerned chiefly with cytogenetic mapping of gene loci and as such is only incidental to the chief objective of this part of this review which is concerned primarily with the state of our knowledge regarding the longitudinal units of organization of these giant dipteran chromosomes.

The first ideas concerning the polyvalent character of the salivary chromosomes were advanced independently by Koltzoff (30) and Bridges (10). Each postulated that chromosomes are composed of two synapsed, longitudinally octopartite, structures and are therefore to be considered as haploid units or bundles with valences of sixteen. Koltzoff referred to the ultimate longitudinal threads, of which he considered the chromosome to be composed, as "genonemata". A genomeme could be considered equivalent to a gene string, or, perhaps a little more accurately, to a string of ultimate chromomeres. He further considered that the bulk of the chromosome is made up of a cylinder of chromoplasm in which the genonemata are peripherally disposed as parallel spiral threads. The conclusion of both these investigators regarding the valency of the chromosomes was based partly on the observation that sixteen "dots, vesicles or small capsular units" (terms used by Bridges) could sometimes be counted in the fine, medium or coarse rings or discs and partly on the deduction that the great size of these chromosomes should logically be explained on the basis of chromonemal reproductions without the usual dissociation of the reproduced elements. The total length of the salivary chromosomes, according to Bridges, is in the neighborhood of 1,180 microns, as compared to an overall length of only 7.5 microns for ordinary gonial chromosomes, the length of the former being therefore about 150 times that of the latter.

In view of the great size difference between salivary and gonial chromosomes Metz (41, 42) and Metz and Lawrence (43) argue that on the basis of the estimate that a single chromonema occupies about one fourth of an ordinary gonial chromosome the extension of such a small structure to the length it would have to occupy in the

corresponding salivary chromosome would result in such a finely attenuated thread that its diameter would be below the limits of microscopic resolution. Metz (42) and his associates argue further that if chromonemata are visible in the salivary chromosomes they would have to be greatly hypertrophied and "... if hypertrophy is admitted the entire enlargement could be explained on that basis alone". He further considers that the postulate that the salivary chromosomes owe their large size to repeated multiplication of chromonemata is based on an assumption which is born of theoretical considerations rather than on direct evidence. To him the longitudinal or diagonal lines, interpreted by others to be chromonemata or bundles of chromonemata, are merely stress lines resulting from the stretching or twisting of the chromosome. His observations indicate that insofar as it has been possible to determine ultimate structure with our present methods, the salivary chromosomes reveal a basically alveolar structural pattern, and that the continuous phase of the material, especially when stretched, is amenable to the false interpretation that it is made up of fundamentally parallel threads.

In contrast to many of the views expressed by Metz and his co-workers, Painter (54, 55) sees in the salivary chromosomes what he considers to be direct evidence for the reality of the chromonemata and the polytene nature of the chromosome organization. The argument that normal gonial chromonemata elongated to salivary dimensions would be invisible microscopically is met with the contention that the threads are probably bundles of chromonemata and are therefore polyvalent themselves, although to a lesser degree obviously than the whole chromosome of which they are its component parts. Painter's conclusions are supported by the microscurgical studies of D'Angelo (2) whose observations with regard to the reality of the threads are at variance with those of other workers (*e.g.*, 11).

It is not within our province here to consider in detail all the bases for the arguments put forth by the respective proponents of the alveolar and of the visible chromonemal structure. No one is better qualified than the proponents themselves. It is therefore suggested that if the reader has any further interest in the more detailed pros and cons of the question of polytene structure and wishes to go beyond the brief and necessarily inadequate picture presented above, he should be directed to read the treatments of the

subject (12, 42, 47, 55). The bibliographies of these papers will guide the reader to the published works of other investigators, not cited here, whose ideas and observations in general support either the alveolar or the chromonemal point of view.

While one can find little in the structural picture presented by Metz and his associates to support the concept that the salivary chromosome is really a polyvalent structure, there is, on the other hand, nothing in this picture which can be regarded as contradictory to such a concept, and there is at least one line of evidence other than direct observation of the chromosomes themselves which supports the interpretation of polyvalency. The measurements by Jacobj (28) of nuclear volumes in the cells of mouse liver revealed to him a rhythmical growth pattern which was the result of successive doublings of the nuclear volume. He interpreted these doublings to be the result of inner divisions of the chromatic material without subsequent nuclear division. Hertwig (21), in consideration of the volume of the salivary gland nuclei in *Drosophila melanogaster*, arrived at the inference that the chromosomes within them have a polyvalency of the order of 256 or 512 as a result of six or seven doublings of the original quadripartite (two synapsed bipartite homologues) arrangement. Lest we attach too much significance to a necessary relationship between nuclear size and chromonemal content, however, we must take into account the observations of Geitler (17). In *Gerris lateralis* (Heteroptera) Geitler was able, by counting the number of heteropycnotic x-chromosomes, to establish a direct relationship between chromonemal content and nuclear volume in many instances, but he was able also to demonstrate cases where the nuclei remained diploid, according to his count of the x-chromosomes, and yet underwent an increase in nuclear volume which was associated with an increase in the amount of nuclear sap. Painter's (54) investigations include the tracing of the development of the salivary gland nuclei from small nuclei in which the chromosomes are optically quadripartite. But even with such an approach, the limitations of our present microscopic technique leave unanswered many questions regarding the finer structural details. Painter, in recognition of these limitations, admits "That the internal arrangement of the ultimate chromomeres in a spiral is an inference based on indirect evidence for we have been unable to detect it by direct observation".

Some new interpretations regarding the polytene nature of the salivary chromosomes have recently been advanced by Ris and Crouse (58) and Hinton (23). The observations of Ris and Crouse have led them to consider that "The giant size of these chromosomes would then be due to: (1) great increase in the length of the chromonema (longitudinal growth of individual genes); (2) increase in the number of chromonemata by endomitosis; (3) lateral separation of the coiled chromonemata". Hinton sees no optically demonstrable chromonemata and doubts the reality of such threads in the interband areas, but he clings to a modified version of polyteny when he attributes the visible expression of a band to numerous duplications of the gene molecule.

ENDOMITOSIS

The observations of Geitler (17) on the origin of cells having multiples of the somatic number of chromosomes in *Gerris lateralis* led him to an analysis of the process for which he used the term "endomitosis". On the basis of changes in the chromosomal behavior he described endomitosis in terms of "endoprophase", "endometaphase", "endoanaphase" and "endotelophase", and pointed out that there is a great deal of similarity between this process and ordinary mitosis. There exist, however, two important differences. In endomitosis there is no strong metaphase contraction of the chromosome. Furthermore, even though endomitosis is characterized by chromonemal and chromosomal reproductions which are in turn followed by separation of the reproduced elements, this separation is not an active dicentric anaphase movement but merely a passive falling apart, and the whole reproductive process takes place within an intact nuclear membrane. Each endomitosis is therefore responsible for a doubling of the somatic chromosome number, and repeated endomitoses in the same cell are responsible for higher multiples of this number. By counting the number of x-chromosomes Geitler was able to establish the presence of such multiples in Malpighian tubules, testicular septae, follicular epithelium, fat bodies, cells designated as oenocytes, salivary glands, connective tissue and gut epithelium. He estimated that some of the salivary gland cells were at least 1024-ploid (512-somatic), indicating nine successive reproductions of an original pair of homologous chromosomes or of the original monosomatic number of chromo-

somes. This is especially interesting, since this value is consistent with some of the estimates of the degree of polyvalency of dipteran salivary chromosomes. It is therefore worth emphasizing that physiological and morphological differentiation in both instances seems to be associated with repeated chromonemal reproductions within an intact nuclear membrane, the chief difference being in the formation of large polytene chromosomes with the retention of the original somatic (or in consideration of synapsis, haploid) chromosome number in the Diptera as opposed to the establishment of multiples of the monosomatic chromosome number in *Gerris*.

Painter and Reindorp (56) have described stages in the nurse cells of *Drosophila melanogaster* in which they observed chromosomal behavior essentially similar to that described by Geitler. In these cells chromosomal reproductions occur repeatedly until 512-ploidy is established. Variations in the staining of the chromosomes at different stages have inclined them to consider also the possibility that similar variations in the staining of the salivary chromosomes might be indicative of reproductive changes in connection with the establishment of the polytene structure. Recently Mickey (45) reported endomitosis in several tissues of *Romalea microptera* (Orthoptera).

In plants the only recorded case of endomitosis in the restricted sense of Geitler seems to be that described by Witkus (66, 67) in the tapetal cells of *Spinacia oleracea*. Coincident with meiosis in the microsporocytes, the chromosomes of the tapetal nuclei undergo a reproduction which is followed by mitosis that in some cases is incomplete so that cells either with two nuclei or with a single dumbbell-shaped nucleus are formed. In these nuclei a second endomitotic chromosomal reproduction occurs which results in a doubling of the chromosome number without breakdown of the nuclear membrane or establishment of a spindle.

How extensive is the occurrence of endomitosis in the plant and animal kingdoms depends upon which definition of the term one accepts. As Grell (19) has pointed out, there is as yet no justification for the assumption that endomitosis, as defined by Geitler, is responsible for supernumerary chromonemal reproductions in the dipteran salivary gland cells or in the cells of the mosquito ileum. With the possible exception of the tapetal cells of *Spinacia* it is likewise true that in the plant kingdom where supernumerary chromonemal re-

productions have been responsible for the occurrence of multiples of the somatic number (*e.g.*, 7, 16, 18, 32, 37, 39), there is no demonstrable series of stages (endoprophase, endometaphase, *etc.*) such as Geitler describes in some detail.

The marked tendency on the part of many recent investigators to use the term "endomitosis" to apply to all cases where repeated chromonemal reproductions occur within an intact nuclear membrane is not, however, without some justification. This tendency reveals the need for some single term which will include all the intranuclear phenomena which this reviewer, for want of a better expression, has classified under the admittedly awkward designation of "supernumerary chromonemal reproductions". Perhaps usage will determine a more extended coverage for the term "endomitosis".

MULTIPLE CHROMOSOME COMPLEXES

In the larval ileum of the mosquito, *Culex pipiens*, Holt (24) described dividing cells which contained various multiples of the somatic chromosome number. In a further study of these multiple complexes Berger (5, 6) described a unique and interesting situation. In the larval ileum the single layer of epithelial cells grows as a result of an increase in cell size and not in cell number. These cells remain in resting stage during larval development and there is no evidence during this period, other than the size increase itself, that there is a reproduction of the chromonemal complex. Certainly there are, according to Berger, no endomitotic stages of the type described by Geitler and by Painter and Reindorp. At the beginning of pupal metamorphosis these cells do not undergo cytolysis, as was formerly supposed, but they finally emerge from the resting stage and undergo a series of divisions. It is possible to count the chromosomes which appear at metaphase of the first of these divisions and thus ascertain the extent of the chromonemal reproductions which occur during the long so-called resting stage. Inasmuch as all the resting cells are not the same size it was possible for Berger to make a correlation between the size of the cell and the number of multiples of the somatic chromosome number which appeared at this time. In this and later divisions the chromosomes arrange themselves in pairs, the individual members of which separate dicentrically at anaphase until the chromosome number

(successively halved at each division) again reaches six, the normal somatic number. Since Berger reported chromosome numbers as high as 96, it follows that there was a four-fold doubling of the original six. As in meiosis, where polyvalent aggregations of chromonemata are also present, successive cell divisions occur without intervening growth periods, this being consistent with Hertwig's (22) ideas on the subject.

Later studies by Grell (19) on *Culex* confirmed the observations of Berger regarding the origin of the multiple chromosome complexes. She was unable to bring to light any direct observational evidence for an endomitotic process, such as that described by Geitler. The appearance of multiples of the somatic number of chromosomes during the long so-called resting stage and the increase in the size of the cells during that period allow only the conclusion that chromonemal reproductions occurring within an intact nuclear membrane (endomitosis in the broader sense) have been responsible for the occurrence of these multiples. Another important consideration in support of this conclusion is that in the prophase immediately following this long resting stage these multiples of the somatic number, however large, are organized into six bundles, the multiple complexes, which because of this grouping indicates that each bundle originated from a single chromosome.

The unique aspects of the situation in *Culex* may therefore be summarized briefly. The chromonemata reproduce repeatedly apparently during the resting stage, while the nuclear membrane remains intact. These chromonemata remain grouped together and can be resolved into bundles of chromosomes during the prophase immediately following. That these bundles arise by repeated doublings in the resting stage is manifest from the fact that the number of constituent chromosomes is always represented by an exponential power of two. The bundles fall apart into pairs of chromosomes. Dicentric anaphase movement separates the members of each pair, and simultaneously sister (or homologous?) chromosomes become again associated in pairs, the somatic pairing (Grell). This paired association becomes progressively more intimate through the succeeding late anaphase, telophase and interphase, and in the following "later division" (Grell) prophase a degree of synapsis somewhat less than that occurring at meiosis is demonstrable. The members of such synapsed pairs again dis-

sociate at anaphase, and the processes of pairing, synapsis and dissociation are repeated in this manner through the succeeding nuclear division cycles until the basic somatic chromosome number is reestablished.

It may be significant to call attention to the consideration that the organization of the chromonemata or chromosomes into bundles may represent a condition which is intermediate between the postulated polyvalent chromonemal association in dipteran salivary gland chromosomes and the complete dissociation of the chromonemata which results in the ultimate somatic doubling of the chromosome number in certain tissues of animals and plants where no bundles are formed but where a residual somatic pairing of sister chromosomes is manifest to a greater or lesser degree.

POLYSOMATY

Usage so far seems to have dictated application of the term "polysomaty" almost exclusively to the situation in many plants where, coexisting with normal (monosomatic) cells, cells containing multiples of the somatic number are of sporadic or of regular occurrence. That the term "polysomaty" should be limited to the plant kingdom seems unjustified, for it could be equally convenient and descriptive of the situation in animals where such multiples occur in association with normal monosomatic tissues.

Although the literature is replete with isolated instances of somatic doubling of the chromosome number in animals and plants where polysomatic cells have originated either spontaneously or as a result of experimentation, it is not our purpose here to compile a catalogue list of such cases. Rather are we concerned with the development of the concept of polysomaty, especially in such cases where it has been demonstrated that the polysomatic cells are the result of supernumerary chromonemal reproduction or of endomitosis in the broad sense of that term.

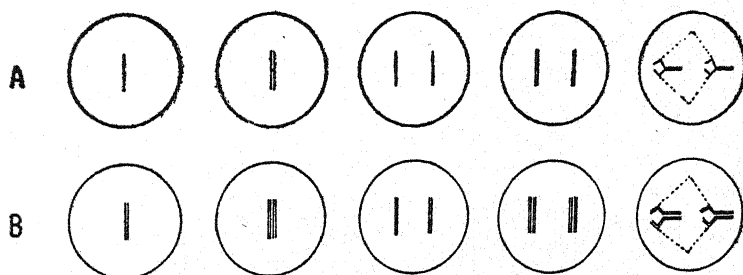
Chronologically treatment of the growth of our knowledge of the subject properly begins with the work of Stomps (61). Although the literature records earlier instances of the existence of polysomatic cells under some other designation than that employed by Langlet (32), the work of Stomps on *Spinacia oleracea* proved to be the starting point for a series of investigations on this and other plants, each of which contributed something to the gradually devel-

oping awareness that naturally occurring polysomaty in the Angiospermae, especially in the Dicotyledonae, is a comparatively common phenomenon and that the polysomatic cells owe their origin in most cases not to nuclear fusions, restitution nuclei, endoduplication (29) or to similar phenomena, as was formerly supposed, but to the occurrence of a supernumerary chromonemal reproduction in an otherwise normal mitotic cycle. The observations of Stomps on the chromosome numbers in *Spinacia* have been confirmed many times by later workers, but his explanation that the syndiploid (disomatic) cells arise by "Kernverschmelzung" has since been rejected in the light of further study.

It was not until the year 1923 that de Litardière (37) demonstrated that the hypothesis of nuclear fusion was inadequate to account for the presence of the strikingly paired chromosomes in many disomatic cells. To this investigator must go the credit for what is perhaps the most significant contribution to our knowledge of the method of somatic doubling, for it was he who first posted the occurrence of a double reproduction of the chromosomes to account for the origin of polysomatic cells. De Litardière and later workers were able to demonstrate that although the double nature of chromosomes is often difficult to observe in normal monosomatic prophase, in many large cells of the periblem doubleness is easily manifest. In fact in some cases the reproduced elements were widely separated, although they still maintained a paired relationship, and it became possible in late prophase and early metaphase to demonstrate the presence of a second reproduction by the frequently diverging ends of the chromatids. De Litardière was the first to realize that the prophase with paired threads were the precursors of metaphases where, instead of the usual 12 chromosomes each with the usual division into two chromatids, there were 24 chromosomes arranged in 12 pairs, and the division of each member of each pair into its component chromatids could be observed in many instances. The paired threads in prophase, as shown by later workers, were in many instances individually bipartite. It therefore became established that the visibly double arrangement was in reality a quadripartite arrangement in which a separation of the reproduced elements at the plane of the first reproduction became the major division into two chromosomes, and the plane of the second reproduction became the one which divided each chromosome into its

two component chromatids. It was postulated by de Litardière that both of the reproductions which were responsible for disomatic metaphases with paired chromosomes occurred in the immediately antecedent prophases which, according to him, were of very long duration, as compared to normal monosomatic prophases. He was unable to cite, however, any experimental evidence in support of the postulate that the prophases responsible for the establishment of polysomatic cells were of any longer duration than normal prophases.

In an attempt to clear up this point Lorz (39) was unable to confirm or deny this postulate. His determination of the ratio of the number of prophases to metaphases in fixed root tips, taken by him to be an indication of the relative lengths of these two stages, revealed that the prophase-metaphase ratio was slightly (perhaps not significantly) greater where the chromosomes were unpaired than where they were paired. Were we to grant de Litardière's postulate that the double reproductive prophases are of very long duration, then the duration of the metaphases with paired chromosomes would be correspondingly long, and if this were true one could argue that the whole nuclear cycle is longer than normal where polysomatic cells arise by a double chromonemal reproduction. According to de Litardière, both reproductions occurred in the prophase in a manner illustrated schematically in the following figure. Endomitosis as described by Geitler would not be very different from the process of doubling as described by de Litardière.



A schematic representation of the sequence of the reproductive processes responsible for a doubling of the chromosome number applied to: (A) a postulated originally single thread from the preceding anaphase; (B) a postulated originally double thread from the preceding anaphase. As to the position of such reproductive phenomena in the mitotic cycle see various interpretations in the text.

Both de Litardière and Langlet (32) emphasized that the paired association of chromosomes in polysomatic mitotic figures was not

the result of an active synaptic affinity between homologues but rather the consequence of the mode of origin, and the latter especially pointed out that in *Cannabis sativa*, where Breslawetz (8, 9) had attributed the cause of polysomaty to nuclear fusions, the paired association of the chromosomes was convincing evidence that polysomatic cells were the result of a double reproduction, as illustrated in the accompanying figure. In a strict sense degrees of pairing are therefore not degrees of association but degrees of dissociation.

The existence of polysomatic metaphases which exhibited little or no pairing of the chromosomes was noted by de Litardière, and his contention that these cells had lost the paired association in passing through one or more nuclear cycles without further doubling was subscribed to by later investigators. In this connection Lorz (39) outlined the following sequence of metaphases in the establishment of the higher multiples of the somatic chromosome number: " $2n$ chromosomes unpaired, $\rightarrow 4n$, paired, $\rightarrow 4n$, unpaired, $\rightarrow 8n$, paired, $\rightarrow 8n$, unpaired, $\rightarrow 16n$, paired, \rightarrow perhaps $16n$, unpaired".

The contention that very long prophases alternate with prophases of normal duration in order to produce the succession of metaphases just described was considered somewhat inadequate by Lorz. On the grounds that such an alternation would involve a certain discontinuity of development and on the grounds that it is possible to demonstrate in polysomatic cells varying degrees of association from close pairing to random scattering in any given stage where the chromosomes are visible, Lorz postulated that a continuously developing asynchronous relationship between the cycle of chromosome reproduction and that of cell reproduction could better account for the origin of the polysomatic cells. On this hypothesis the cycles of chromonemal reproduction and nuclear division mechanics lose their normal interdependence, and if the cycle of the former proceeds at a more rapid rate than that of the latter, the eventual occurrence of two chromonemal reproductions within one nuclear cycle is the result.

Gentcheff and Gustafsson (18) believe that both reproductions of the chromosomes associated with the origin of polysomatic cells occur at a stage prior to early prophase because they have been able to demonstrate the existence of pairs of parallel bipartite threads (quadrivalent associations) at the earliest visible stages. Berger (7) confirms this observation but states definitely his

belief that both reproductions occur in the resting stage and draws a parallelism between this situation and the situation in *Culex* where the evidence points to the interpretation that the multiple chromosome complexes owe their origin to "repeated chromosome division without mitotic activity" (see 4, p. 187). These observations are also in accord with those of Lorz (39) who observed pairs of threads (probably individually bipartite but not always optically so) in early prophase. Lorz, however, considers that in many cases where there is observational evidence that only one chromonemal reproduction has occurred prior to early prophase, as in normal monosomatic nuclei, there is no assurance that another reproduction could not have been possible in later prophase, had the subsequent growth of the cell not been prevented by fixation.

The direct observations of Gentcheff and Gustafsson were further supplemented by their experiments involving the x-raying of spinach seeds which were exposed in the dry and dormant condition in order to establish with certainty that all cells were in the resting stage at that time. Upon germination of these exposed seeds, cells from the radicles contained chromosome fragments which followed the same reproductive course as the unaltered chromosomes. In the di- and tetrasomatic metaphases with paired chromosomes they also found paired fragments which exhibited the same degree of chromatid doubleness as the unaltered chromosomes. The conclusion that two effective doublings have occurred after the x-ray treatment of an effectively single thread seems to be well founded, although Berger (7) makes the point that "... until more is known about the condition of the chromosomes in the abnormal nuclei of dry seeds, the evidence for their singleness will remain inconclusive". Berger bases his point on the grounds that cells in dry seeds are in a plasmolysed condition, and he therefore considers that the chromosomes also may be dehydrated to the extent that "... the members of a reduplicated chromosome would be so close together that they would act as a single unit". The implication from this is that if effective doubling in the sense used by Sharp has occurred, a reversion to the status of elementary doubling (effective singleness) may possibly take place in the plasmolysed cells of dormant seeds.

In an extension of the same idea, Berger's objections may not go far enough, for neither he nor Gentcheff and Gustafsson gives

any consideration to the possibility that the extremely low rate of metabolism associated with dormancy may be contingent upon a certain fixed state of organization of the chromonemata in the cells of the quiescent meristematic tissues, a state which may be essential to dormancy but not to the highly metabolic activity of dividing and growing cells. In other words, in a quiescent meristematic cell, reproductive activity of the chromonemata may have to stop at a certain fixed point before the cell can enter into a type of resting stage which is probably as near as possible to a resting stage in an absolute sense. On the other hand, two optically similar so-called resting nuclei from an active meristem may differ from each other in the organization of their not optically resolvable chromonemata.

As is often the case in the development of scientific knowledge, it is not always easy to differentiate fact from conjecture, but the essential facts applying to the process of somatic doubling in *Spinacia*, as they appear to this reviewer, are as follows: (a) As a result of the occurrence of two chromonemal (= chromosomal in this instance) reproductions in one mitotic cycle, cells which were monosomatic become disomatic, and this process may be repeated to produce higher degrees of polysomaty, although normal mitoses may intervene between successive doublings. (b) The appearance of optically bipartite chromosomes arranged in pairs is conclusive evidence that a double reproduction has been responsible for polysomaty. (c) From the x-ray experiments of Gentcheff and Gustafsson it is evident that the double reproduction (effective doubling at least) follows the stage represented by resting nucleus which is also in a state of physiological dormancy. (d) Relational coiling is manifest at times between the two bipartite threads which originate from a single thread through double reproduction. (e) The presence of paired bipartite chromosomes in certain favorable early prophase indicates that in these cases the double reproduction occurred before the chromosomes emerged as visible structures. (f) Different degrees of pairing are manifest in different polysomatic nuclei, ranging from a condition of close pairing through more remote association to complete randomness.

In the light of the above facts the principal theoretical considerations with regard to the time of occurrence of double chromonemal reproduction may be summarized as follows: (a) Both reproductions occur in the prophase. This is the view originally held by

de Litardière (37) and later supported by Langlet (32) without benefit of the knowledge derived from later investigations. (b) Two reproductions occur within one nuclear cycle when the rate of chromonemal reproduction exceeds that of nuclear division. This view, advanced by Lorz (39), may become untenable in the light of the evidence from the x-ray experiments of Gentcheff and Gustafsson if it can be proved that the organization of the threads in the resting nuclei of actively meristematic cells is consistently the same as that demonstrated in the nuclei of the dormant meristems of dry seeds where physiological and reproductive activity is held to a minimum. Later investigators apparently attach no significance to the occurrence of variable degrees of paired association in polysomatic nuclei which Lorz considers supports his hypothesis. (c) Both reproductions occur in the interval between resting stage proper and the earliest discernible prophase. This is the view held by Gentcheff and Gustafsson (18), whose observations on favorable early prophases reveal that a double reproduction has already occurred, but whose x-ray experiments indicate that the thread was effectively single in the dormant resting stage. (d) Both reproductions occur in the resting stage. This is the view held by Berger (7) who bases his conclusion also on the fact that the visible organization of the chromosomes in favorable early prophases indicates that in these cases the double reproductions have already occurred. Berger sees here a situation parallel to that in *Culex* where repeated chromonemal reproductions occur in the resting stage. Ervin (16) also considers that both reproductions probably occur in the resting stage and like others bases his conclusion on the fact that it is possible to observe cases where the organization of the threads in early prophase indicates a prior occurrence of doubling.

While the observations of Berger, Ervin, Gentcheff and Gustafsson and Lorz on early prophases leave little doubt that both reproductions can and do occur at some stage prior to prophase, in many cases there still seems to be a question as to whether the conclusion is warranted that this happens in all cases. Lorz (39), in his figure 9, was unable to demonstrate the existence of the second reproduction, but the parallel disposition of the threads and the remoteness of their paired association indicate strongly that the first at least has occurred and that the parallel threads are not simply

the chromatid halves of a single chromosome. If the second reproduction has occurred in the cell in question it is not optically manifest. If, on the other hand, the second reproduction has not occurred it seems likely from the remoteness of the products of the first reproduction that if the growth of this cell had not been stopped the resolution of each of the paired threads would have become possible in later prophase or at least in metaphase. If the universality of the occurrence of the quadripartite organization of early prophase associations, as depicted by figure 6 of Gentcheff and Gustafsson, can be established, then the hypothesis of asynchronism advanced by Lorz becomes untenable.

Factors Associated with or Responsible for Polysomaty

Where polysomaty is of regular occurrence in plants it is generally agreed by most investigators in the field, especially the later ones, that it is the result of a double reproduction of the chromosomes whose frequently strikingly paired association is unmistakable evidence that this has been the mode of origin. But little is actually known, however, concerning the physiological factors associated with this deviation from the normal mitotic process. Ervin (16), working with *Cucumis*, described slight increases in the frequency of polysomatic cells in the roots of young seedlings as a result of certain heat treatments and certain treatments with indole-3-butyric acid. He also studied the roots of seedlings which had been exposed to the action of colchicine. In the first two cases there was no evidence of any spindle abnormalities, but the colchicine treatment apparently produced some polysomatic cells as a result of spindle-inhibition. Levan (34) was able to produce polysomatic cells experimentally in *Allium cepa* through the use of growth-promoting substances. In the absence of any evidence in the nature of spindle irregularities he considers that the doubling of the chromosome number took place in the resting stage. The observations of Dermen (15), which will be considered in greater detail later, in general confirm those of Levan.

The natural occurrence of polysomaty in *Spinacia*, *Cannabis*, *Cucumis* and other genera where this situation has been studied in some detail seems always to be associated with age and differentiation and with an increase in nuclear and cell size. Very actively embryonic and undifferentiated cells are rarely if ever polysomatic.

Where polysomaty seems to be part of the normal developmental processes, as it so appears in the roots of the above-mentioned genera, the cells containing the higher multiples of the somatic chromosome number are found usually in the region of differentiation where the tissues are sharply differentiated into dermatogen, periblem and plerome, the developmental anlage of the epidermis, cortex and stele, respectively. In the periblem, where polysomaty is most frequently encountered, vacuolization has already become established to an appreciable degree, and in the most highly differentiated cells which are still capable of mitosis are found division figures which reveal the highest multiples of the somatic chromosome number.

The experiments of Levan (34), Ervin (16) and Dermen (15) suggest therefore that perhaps the natural origin of polysomatic cells may be due to the elaboration by the organism itself of similar chemical growth-promoting substances. It may well be that the elaboration of such substances in slightly excessive amounts may stimulate real growth (increased amount of protoplasm, not merely intake of water) to such an extent that the developing polysomatic cells exceed the size usually attained by monosomatic cells before division, and coincident with this size excess may be an excess of chromosome growth and reproduction. Otherwise stated, when a normal cell reaches a certain growth endpoint, division follows, but in an abnormally stimulated and rapidly growing cell it may be possible for growth to surpass this endpoint before division is initiated. The chromonemal constituents of such a rapidly growing cell might conceivably complete the one reproduction usually associated with the nuclear cycle and begin another in the realm of elementary doubling while the threads are effectively single and, in stages where they are optically resolvable, also visibly single. It is hardly likely that a complex gene molecule is synthesized in an instant, but rather there are steps (a chain of chemical reactions) in the process.

That increase in cell and nuclear size is usually associated with an increase in the number of chromosomes has been a common observation, but Gentcheff and Gustafsson (18) made the interesting observation that polysomatic cells not characterized by a conspicuously paired association of the chromosomes were larger than cells polysomatic to the same degree in which the conspicuous pairing is

evidence of recent origin. They thus have demonstrated that some growth apparently takes place in the absence of further visible double chromosomal reproductions, a fact which seems to indicate that increase in cell size precedes visible doubling, at least, and may precede effective doubling, but whether it precedes, follows or is merely concomitant with elementary doubling is, of course, still undetermined. In normal mitosis the situation is indeed similar, for we are not yet in a position to state the order of precedence, if any, of cell division and the single chromonemal reproduction associated with it. If chromonemal reproduction results from the synthesis of a copy like and alongside the original, then the development or formation of such a copy may be a continuous growth phenomenon accompanying the growth of the cell, and it is even conceivable that before a synthesis of one copy is complete the synthesis of another may be initiated. Thus in terms of ultimate elementary constituents, an optically single thread may not only be effectively double but may instead be effectively single and contain one or more partly developed copies.

With regard to the association of polysomaty with the process of tissue differentiation it is interesting to note that also in the animal kingdom (*e.g.*, Heteroptera, Diptera, and Odonata; 52) where supernumerary chromonemal reproductions have been demonstrated or postulated the cells concerned are not found in undifferentiated embryonic tissues but in tissues which are specialized morphologically and physiologically.

Polysomaty in Plant Neoplasms

Consistent with the fact that polysomaty may be associated with mild growth disturbances is the observation that they are also associated with greater disturbances which result in the formation of plant neoplasms. Jørgensen (29) attributed the origin of tetraploid shoots from callus tissue in *Solanum* to the presence of disomatic cells in otherwise diploid (monosomatic) tissue. According to him, such cells resulted from coalescence of two mitotic spindles of a dividing binucleate organization. Winge (63) and Levine (36) described polysomatic cells in crown gall tumors in *Beta vulgaris*. The former described diakinesis-like prophase and the latter mentions the occurrence of tetrad-like structures at metaphase. From what we now know about the origin of polysomatic cells as a result

of a double chromosomal reproduction it becomes possible to interpret these descriptions in their true light. Levine made the observation that the polysomatic cells occurred in the older portions of the crown gall tumors, a fact which correlates well with the occurrence of polysomaty in the older, more differentiated, part of the root periblem of *Spinacia* and other plants. Wipf and Cooper (65) describe polysomatic cells in the rhizobial tubercles on the roots of certain legumes, but despite their well illustrated instances of paired chromosomes they cling to the interpretation that nuclear fusion is the probable method of origin. Dermen (15), using 0.25% naphthalene acetic acid in lanolin paste, was able to produce tumor-like growths on the stem internodes of bean in which he attributes the origin of tetraploid (disomatic) cells to intranuclear multiplication of the chromosomes and considers the occurrence of paired associations significant.

Incidence of Polysomaty

Although the term "polysomaty" has not been widely accepted to describe multiples of the somatic chromosome number in the animal kingdom, it has been amply demonstrated by the published observations on *Culex* ileum, *Drosophila* nurse cells, various tissues in *Gerris*, connective and fat tissue in the Odonata, etc., that such multiples exist. Since, however, there are so many uninvestigated or incompletely investigated cases in the animal kingdom where cell size considerations have suggested the possible existence of chromonemal or chromosomal multiples it would be difficult to give any near accurate estimate here of the incidence of polysomaty or its equivalent.

Similarly in the plant kingdom, for somewhat the same reasons, it is difficult to present a true picture of the incidence of polysomaty although a larger number of cases have been studied. In some plants where polysomaty has not been definitely demonstrated there is evidence from the large size of the periblem cells that multiplications of the somatic chromosome complement may have occurred but may have escaped detection. This could be especially true in view of the consideration that the polysomaty, in these cases probably only disomaty, of these cells is probably established in the last division before the cells become permanent. Consequently these cells may have passed through only this one division which in consideration of its short duration compared to the resting state

both before and after would therefore be only rarely observed. Accordingly in some instances in the literature where the sporadic (?) occurrence of disomatic metaphases has been indicated by either text figure or description, they have been considered as oddities and hardly as characteristic of the organism. Usually also the origin of these cells has been variously explained in excusable ignorance of the later developed state of our knowledge regarding the facts and interpretations of polysomaty or in failure to recognize the true significance of the conspicuously paired association of the chromosomes. Cases in point are those in *Solanum tuberosa* (57), *Arachis hypogaea* (27) and *Acer platanoides* (44). Doubtless, as time goes on, more cases of polysomaty will come to light.

With respect to the location of polysomatic cells in plant tissues the published results indicate that they occur most frequently in the periblem of the root. Certainly they are the most easily demonstrable there. Their occurrence in the plerome is relatively rare but has been reported in *Spinacia*, *Cannabis* and *Cucumis*. Their occurrence in plant neoplasms has already been considered. Moffett (46) described their presence in the flowering spikes of *Knophofia*, and they have been reported in the tapetum of *Spinacia* (67) and *Galtonia* (60), although in these two latter cases polysomaty is the result either of endomitosis in the more limited sense of the term or of the coalescence of two mitotic spindles in binucleate cells. It is significant that in most cases polysomaty is not manifest in cells which undergo meiotic reduction into spores or gametes. Otherwise, naturally occurring polyploid forms would be much more frequent than they are.

Practically all cases of polysomaty in plants which have come to the writer's attention have been observed in the Angiospermae. Whether this condition occurs with any frequency outside this class remains to be demonstrated. Without representing an exhaustive compilation of all cases where polysomatic cells have been described, the accompanying table gives the reader an overall view of the situation in the Angiospermae with regard to families and genera where polysomatic cells have been reported. In view of the consideration that in certain instances earlier interpretations with regard to the origin of polysomatic cells have been shown by later work to be in error, no concerted attempt has been made in the preparation of the table to differentiate those cases where polysomaty

has been shown to be due to an intranuclear double reproduction of the chromosomes from those where some other method of origin has been either demonstrated or postulated. However, in most cases marked by a dagger in the table, especially in *Spinacia*, *Cannabis* and *Cucumis*, it is established that polysomaty is of regular occurrence and that its origin is the result of an intranuclear double reproduction of the chromosomes. Many special cases where the evidence seemed clear cut that a sectorial polysomatic chimaera probably arose only once from a single initial cell were purposely excluded from the table, since we are chiefly concerned with cases where polysomaty occurs to a greater or lesser degree as a regular developmental process or where observations and experiments suggest that an inherent though latent tendency toward regular polysomaty may be present. It was felt that such special cases had little to contribute to the general understanding of the underlying phenomena.

THE INCIDENCE OF POLYSOMATY IN THE ANGIOSPERMAE

GENUS	FAMILY	AUTHORITY*
<i>Ranunculus</i> †	Ranunculaceae	Larter (33)
<i>Brassica</i>	Cruciferae	Netroufal (51)
<i>Gibbaeum et al.</i>	Aizoaceae	Wulff (69)
<i>Spinacia</i> †	Chenopodiaceae	Stomps (61), Berger (7)
<i>Chenopodium et al.</i>	Chenopodiaceae	Wulff (68)
<i>Kochia</i>	Chenopodiaceae	Wulff (68), Lorz (39)
<i>Beta</i> †	Chenopodiaceae	Nemec (49), Levan (35)
<i>Cucumis</i> †	Cucurbitaceae	Ervin (16)
<i>Arachis</i>	Leguminosae	Husted (27)
<i>Pisum et al.</i>	Leguminosae	Wipf and Cooper (64)
<i>Phaseolus</i>	Leguminosae	Dermen (15)
<i>Cannabis</i>	Cannabinaceae	de Litardière (38), Breslawetz (9)
<i>Acer</i> †	Aceraceae	Meurman (44)
<i>Nicotiana</i>	Solanaceae	Webber (62)
<i>Solanum</i>	Solanaceae	Winkler (64), Prokofieva-Belgovskaja (57)
<i>Allium</i>	Liliaceae	Nemec (49), Levan (34)
<i>Gallium</i>	Liliaceae	Smith (60)
<i>Kniphofia</i> †	Liliaceae	Moffett (46)
<i>Sorghum</i>	Gramineae	Huskins and Smith (26)

* Where two or more publications have been concerned with a particular genus an attempt has been made to cite the earliest and the latest.

† Genera where the regular occurrence of polysomaty seems well established.

CONCLUDING REMARKS

In the preparation of this review an effort has been made to limit the subject matter to the more fundamental considerations with regard to intranuclear phenomena which are responsible for

deviations from the normal of either the valence or the number of chromosomes. An attempt has been made to organize under a single heading the consideration of all diverse expressions which are the result of the interplay of three variables of a single fundamental reproductive process. These variables are (*a*) the number of chromonemal reproductions, (*b*) the degree of dissociation of the reproduced chromonemata, and (*c*) the frequency of mitosis. For example, if the number of chromonemal reproductions (variable *a*) is one and the frequency of mitosis (variable *c*) is zero, either the result is a nucleus with a doubled chromosome number or, if the number remains the same, the chromosomes have a doubled valency (expression of variable *b*). In dipteran salivary glands, if the reality of polyvalency be admitted, variables *b* and *c* are at zero, while the degree of polyvalency depends on the expression of variable *a*. In the *Culex* ileum variable *c* remains at zero (the long resting stage), while variable *a* expresses itself in a number of reproductions. After the long larval resting stage variable *a* remains at zero and *c* expresses itself in a number of (in this case reductional) nuclear divisions. Variable *b* finds expression as chromosome complexes and individual chromosomes. In *Gerris* variable *c* remains at zero, variable *a* expresses itself in a number of chromonemal reproductions and variable *b* expresses itself in the dissociation of the reproduced elements which doubles the number rather than the valency of the chromosomes at each reproduction. In *Spinacia* and other similar cases variable *a* may alternate as one, two, one, two, etc., while variable *c* remains consistently at one. Variable *b* finds expression as in *Gerris*.

With regard to the usage of some of the terms a few final remarks seem advisable. From the standpoint of priority of description the term "endomitosis" ought to be strictly limited in both its meaning and its application to cases, of which there are not more than four or five in the literature, where there are a series of optically resolvable stages of chromosomal behavior which can be described as endoprophase, endometaphase, etc. It is possible that even in some of these cases a point for point comparison with the original description of Geitler might reveal differences in detail which might raise the question of propriety in the application of the term. Since convenience is one of the chief reasons for the establishment of a descriptive terminology, however, it would seem that a broader mean-

ing and application of the term is justified. "Endomitosis" has already been used either designedly or unwittingly by a number of investigators in such a broader sense to cover a behavior pattern basically similar in all probability to the stages described by Geitler but applying not only to chromosomes within the range of visibility but to the postulated behavior which is inferred from the organization of the reproduced elements when they do become optically resolvable.

There seems also to be considerable justification for the broad application of the term "polysomaty" and allied terms to describe cells and situations where various multiples of the somatic chromosome number are repeatedly established in a single individual, whether it be plant or animal. This usage seems especially justified, since such terminology emphasizes a regular process of doubling wherein only even and never odd multiples of a basic haploid or n number of chromosomes are possible, at least until it can be definitely established that there actually are cases where one of two genomes becomes double independently of the other. It has been suggested that the triploid tomato shoot of Huskins (25) which developed from diploid tissue might conceivably have had its origin in this manner.

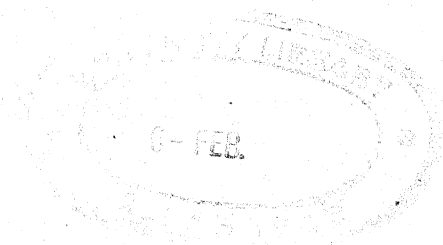
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